

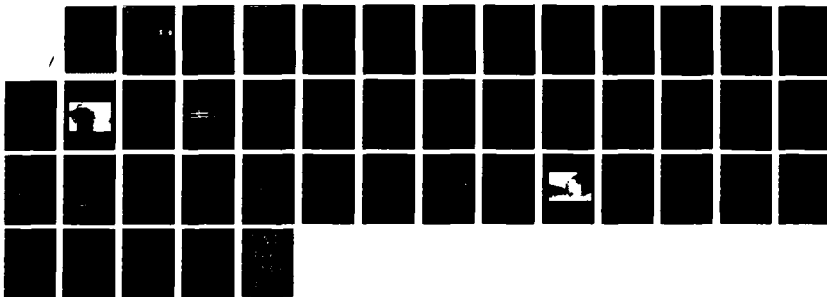
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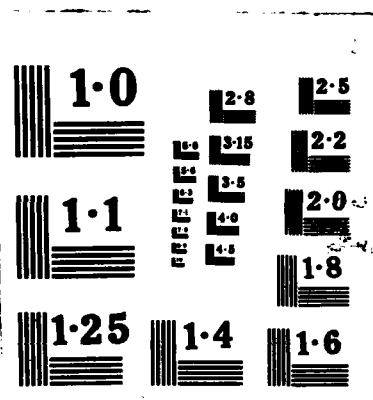
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FEASIBILITY OF HUMAN SKIN GRAFTS ON AN ISOLATED
BUT ACCESSIBLE VASCULAR SUPPLY ON ATHYMIC RATS
AS A SYSTEM TO STUDY PERCUTANEOUS
PENETRATION AND CUTANEOUS INJURY

ANNUAL REPORT

GERALD G. KRUEGER, M.D.

APRIL 1986

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Cont'd → through grafted and nongrafted components of the flap, and have compared absorption across the flap with that which occurs in vitro. These assessments cause us to conclude that this system will be extremely valuable to groups who need accurate assessments of how much of a compound comes across human skin per unit time, and whether it is metabolized as it crosses the skin. Prior to the availability of this flap, these types of analyses simply could not be made in human skin or, for that matter, in animal skin. Keywords: Biological absorption ↑

Summary

The objective of this research was to determine the feasibility of grafting human skin onto congenitally athymic (nude) rats, and to isolate the grafted human skin as a flap of functional skin on an isolated, but accessible, vasculature. This has been accomplished. Thereafter, the proposed system was to be characterized as to structure and function of the skin, and to be validated as a system for studying percutaneous absorption. This report details the progress made during the third year of this feasibility project.

Developing technologies demand change in technique until acceptable rates of success are achieved. Over the past year, our success rate of rat-rat or human-rat skin flaps reaching experimental stage has stabilized. Currently, approximately 50-60% of flaps that are initiated reach the experimental stage. Further, the likelihood of a flap being re-utilized on second and third occasions has increased. Because our technology has stabilized, this annual report details the methodology for generating the flap.

Further analysis of blood flow to the flap has been made along with a correlative analysis of the blood flow to the host side and grafted side of the flap. Analysis of the blood flow to the flap as a function of flap age, as well as the effect of cyclosporine, has also been made. Data demonstrate that blood flow to the graft and host sides of the flap do not differ substantially. The data also demonstrate that the blood flow to the flap does not change significantly over the period of observation (four months). Considerable variability is noted in blood flow in any particular flap on a week to week basis. This is unexplained, but is not related to administration of cyclosporine, body temperature, or age of the flap.

Previously, we have demonstrated that percutaneous absorption of benzoic acid is proportional to blood flow to the skin. This has been confirmed by first noting that rates of absorption on days with high blood flow are higher than those on days with low blood flow, and that this is not related to age of the flap. In addition, it has been noted that percutaneous absorption of benzoic acid through a site that is artificially vasoconstricted with phenylephrine has a considerably different flux profile than skin that is similarly treated with water.

A series of validation studies has been conducted, comparing the absorption of caffeine across the rat graft and rat host component of a rat-rat skin flap. These results have been compared to absorption across the human component of a human-rat skin flap. As a final analysis, in vivo and in vitro absorption of caffeine has been determined using the same human skin source. These data demonstrate no essential difference between the absorption profile of rat graft skin and that of rat host skin. However, the amount of caffeine absorbed across rat skin is approximately 10-fold that across human skin. The comparison of human skin, in vivo vs. in vitro, demonstrates dramatic differences. Flux across human skin in vivo occurs much earlier and is much greater than that which occurs in vitro. This difference appears to be largely secondary to binding in the dermis in the in vitro situation.

We conclude that this model system meets the objectives of the original proposal and will be a significant tool for those groups concerned with kinetics of percutaneous absorption, metabolism of percutaneously applied compounds, and toxic responses generated by topically applied compounds.

Foreword

In conducting the research described in this report, the investigators have adhered to the "Guide for the Care and Use of Laboratory Animals" as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication (NIH 78-23), Revised 1978.



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TABLE OF CONTENTS

	<u>Page</u>
Overall Objectives/Statement of the Problem	9
Background	9
Materials and Methods	
Animals	10
Materials/instruments	10
Blood flow analysis	11
Description of the model	11
Generation of skin sandwich flap	11
Accessibility of vasculature supplying skin flap	14
Dosage of cyclosporine to maintain human-rat flap	14
Percutaneous absorption using the skin sandwich flap	16
Percutaneous application of benzoic acid	16
Percutaneous absorption of caffeine across human, rat host, and rat graft skin	16
Assessment of skin distribution of radioactive compound at the end of the collection of blood sample phase of an experiment	16
Theoretical considerations and calculations	18
Orthotopic transplantation of split-thickness scalp grafts	18
Results/Discussion	
Blood flow in the flap	19
Correlation of actual blood flow with LDV and confirmation of LDV with dermal fluorometry	19
Comparative analysis of blood flow to the flap as a function of age, graft vs. host, and effect of cyclosporine	20
Influence of blood flow on percutaneous absorption of benzoic acid	26
Comparison of absorption of caffeine, rat skin vs. human skin, on the rat skin flap, and in vivo vs. in vitro using human skin	30
Comparison of absorption of caffeine, rat host, rat graft, and human graft on the rat skin flap system	30
Percutaneous absorption in vivo vs. in vitro	30
Binding of caffeine in vivo vs. in vitro	30
Tissue binding, rat-rat and human-rat skin sandwich flaps following the deposition of 36 ug/cm ² of ¹⁴ C-caffeine	33
Assessment of feasibility of transplanting hair-bearing skin to the nude mouse	36
Animals and experimental statistics	38
Conclusions	41
Future Objectives	42
Literature Cited	43
Distribution List	45

Tables and Figures:

Table 1:	Blood Flow Ratios in the Skin Sandwich Flap	23
Table 2:	Percutaneous Absorption of ^{14}C -Caffeine in Ethanol Following Deposition of One and Two Doses on the Human Side of a Human-rat Skin Flap In Vivo	36
Table 3:	Animal Statistics	40
Figure 1:	Schematic Diagram of Generation of a Rat-rat or Human-rat Skin Flap on the Congenitally Athymic (Nude) Rat	12
Figure 2:	Photograph of a Rat-rat Skin Sandwich Flap on a Congenitally Athymic Nude Rat	15
Figure 3:	Schematic Diagram of a Sandwich Island Flap	17
Figure 4:	Correlation of LDV Values with Measured Blood Flow to the Flap	21
Figure 5:	Correlation of LDV Values with Clearance of Fluorescein from the Skin as Measured with the Dermal Fluorometer (DF)	22
Figure 6:	Average Blood Flow As a Function of Flap Age	24
Figure 7:	Blood Flow of the Graft Side vs. the Host Side of the Flap	25
Figure 8:	Influence of Blood Flow on Percutaneous Absorption Profiles of ^{14}C -Benzoic Acid Across the Rat Graft With Flap Age	27
Figure 9:	Influence of Vasoconstriction on Flap Blood Flow in Micro- circulation of the Rat-Rat Flap	28
Figure 10:	Effects of Iontophoresis on Percutaneous Absorption of ^{14}C - Benzoic Acid	29
Figure 11:	Comparison of the Absorption of Caffeine	31
Figure 12:	Percutaneous Absorption of a Single Dose of ^{14}C -Caffeine	32
Figure 13:	Comparison of Application of a Standard Dose of ^{14}C -Caffeine	34
Figure 14:	Analysis of Location of Binding within Rat and Human Skin Components of the Skin Sandwich Flap	35
Figure 15:	Photograph of Hair Growth in a Human Split-Thickness Skin Graft on a Nude Rat	37

Overall Objectives/Statement of the Problem

The objective of this research has been to determine the feasibility of grafting human skin onto the congenitally athymic (nude) rat and to isolate the grafted human skin as a flap of functional skin that is on an isolated but accessible vasculature. Once this objective was accomplished, the proposed system was to be characterized as to the structure and function of said skin, and finally to be validated as a system for studying percutaneous absorption. This is the third annual report of the progress made in pursuit of the foregoing goals between 1 September 1984 and 31 December 1985. Note: The third annual report covers a 15 month period because of a granted request for a 3 month extension of funding. The final report will be forthcoming.

Background

Percutaneous absorption involves a series of sequential transport processes (1). Compounds that are absorbed, traverse the stratum corneum, the epidermis, and the papillary dermis until the superficial vasculature (capillary plexus) is reached. At that point, the compound and/or its metabolites either remain in the dermis or are transported to the rest of the body via the circulatory system. An accurate assessment of this dynamic process in skin in the in vivo state, as a function of time, has been unavailable. Recently, our laboratory embarked upon a project designed to generate a viable skin sandwich flap on the congenitally athymic (nude) rat, which is unique because the flap would exist in a functional state on an independent but accessible vasculature.

The need for a more accurate assessment of absorption, metabolism, and compartmentalization of topically applied agents and how the microcirculation of skin affects these processes in the in vivo state has been fueled by the need to understand the local toxic and/or therapeutic effects of agents which are absorbed through and possibly metabolized by the skin before being transported systemically. Heretofore, to assess percutaneous absorption of a topically applied agent in human subjects, investigators have had to rely on the amount of a topically applied agent that appeared in the blood, urine and/or feces. While the amounts appearing in these various body fluids and secretions have been useful for understanding generalities about percutaneous absorption, definitionally they cannot accurately represent local events in the skin. To circumvent the problem of local events, investigators have turned to in vitro models to study percutaneous absorption.

The currently available in vitro models, using split-thickness human skin, that are utilized to study percutaneous absorption have only been partially verified with in vivo analogues (2). The in vitro models are represented by two types, those that use non-viable skin and those that use "viable skin" with various media support systems. It is assumed, in the latter, that the media support systems represent the normal biologic support systems inherent to mammals. In neither, however, is there an active blood supply to transport the agent or its metabolite from the test site. These systems depend on simple diffusion through the skin into a receiving chamber. Conclusions about absorption and metabolism in such systems, where tissue viability decreases as the experiment continues, demand confirmation in a viable model with an established functional vasculature. As noted, we have developed an in vivo model system which provides the investigator with functional viable skin and

direct access to its supplying vasculature. The principal objective of this feasibility project was to create a system which would permit a direct assessment of transdermal flux and metabolism, as well as monitor possible injurious events that occur following the topical application of compounds on viable human skin without having to resort to direct testing on human subjects. We felt that this objective could be accomplished by grafting human skin onto congenitally athymic (nude) rats and to isolate this skin as a flap of viable, functional skin on an isolated but accessible vasculature. During year 1, we confirmed that nude rats, contrary to nude mice, are capable of immunologically rejecting human skin (3, and unpublished observations). This led to a series of experiments to circumvent graft rejection by nude rats. As noted in our second annual report, this rejection appears to be humorally mediated and can be prevented with low dose cyclosporine.

In the final year of this proposal to develop such a system, we have directed our attention to the following: a) To present a detailed and updated presentation of the methodology used to generate rat-rat, as well as rat-human, skin flaps on an isolated but accessible vasculature on the nude rat. b) To accurately assess blood flow in the flap system and determine how blood flow changes as the flap ages as well as how blood flow alters percutaneous absorption. c) To further validate the system by comparing the absorption of caffeine across the grafted side vs. the host side of rat-rat flaps, to compare these data with those across human grafts, and to conduct similar experiments comparing in vitro absorption vs. in vivo absorption, rat-human skin flap vs. human skin on a Franz chamber. d) To develop a technology to quantitate and localize tissue binding of topically applied compounds. e) To determine the feasibility of grafting adnexal structure-rich human skin to the nude rat. The latter is critical to those situations where a different profile of percutaneous absorption is likely to be influenced by such structures. Each of the foregoing, i.e., the effect of blood flow on rate of percutaneous absorption, the differences in absorption between rat and human skin, and human skin in vitro vs. in vivo, as well as the difference in absorption through adnexal bearing skin, is of importance to situations where rate of percutaneous absorption and altering the rate of percutaneous absorption are important. It follows that all of these components are important to chemical defense.

Materials and Methods

Animals

Outbred nude rats have been used because of their depressed immune system (4,5) and because the partial to complete hairlessness of these rats obviates the need for removal of hair with chemicals or clipping which can result in damage to the stratum corneum. Initial breeding pairs were obtained from the animal production facility of National Cancer Institute (Frederick MD). This local colony was expanded by mating male rats homozygous for nude with female rats heterozygous for nude in the manner previously described for expansion of colonies of nude mice (3). Typically, experimental rats weigh between 200-300 grams at the initiation of experiments.

Materials/instruments

Ketamine, 100 mg/kg, injected intraperitoneally (I.P.), (Ketaject, 100 mg/ml, Parke Davis, Morris Plains NJ) is used to anesthetize the animals for surgery. Additional doses (approximately 10 mg) are administered to sustain anesthesia as needed. Hypovolemia and shock are lessened with the I.P.

administration of 3 ml of bacteriostatic sodium chloride (USP, 0.9%, Elkin-Sinn Inc., Cherry Hills NJ) at the beginning of the experiment. Instruments that are used include: dermatome (Brown Electro-Dermatome, Model 666, Zimmer Inc., Warsaw IN); operating microscope, (Model OPMI 6-SD Carl Zeiss, West Germany) equipped with a fiberoptic illuminator (Model 310187, manufactured for Zeiss by Dyonics, Andover MA); and a Malis bipolar coagulator (Codman and Shurtell Inc., Randolph MA). Disposable materials that are used include: suture, 5-0 Ethilon black monofilament nylon with a PS-2 needle (Ethicon, Inc., Somerville NJ) and a 10-0 black monofilament nylon with a TE 70 needle (Davis + Geck, Inc., Maranti, PR 00701); Dermiform hypo-allergic knitted tape (Johnson and Johnson Products Inc., New Brunswick, NJ); Kling conforming gauze bandage (Johnson and Johnson Products Inc., New Brunswick NJ); Heparin (Sodium injection, USP 1000 USP units/ml, Elkin-Sinns Inc., Cherry Hills NJ); ^{14}C -Benzoic acid (Specific activity 56.8 or 19.3 mCi/mM, New England Nuclear, Boston, MA); Parafilm (American Can Co., Greenwich, CT); Holliseal skin barrier (Hollister Inc., Libertyville IL); sterile Vaseline petrolatum gauze (Chesebrough-Ponds Inc. Hospital Products Division, Greenwich CT); Webcol sterile alcohol wipes (Kendall Company Hospital Products, Boston MA).

Blood flow analysis

Blood flow is assessed non-invasively using a laser Doppler velocimeter (LDV; LD 5000 Med Pacific, Seattle WA) (6-8) or a hand held dermofluorometer, FluoroscantTM (Santa Barbara Technology, Santa Barbara CA) (9,10). Electro-magnetic flow probes (type C with a 0.5 mm diameter, Micron Medical, Los Angeles CA) directly attached to the experimental vessel are used to measure blood flow through the vessels supplying and draining the skin sandwich.

Description of the model

The experimental model consists of a skin sandwich which is generated as a flap by grafting a split-thickness skin graft (STSG), human or rat, .4 mm in thickness, to the subcutaneous surface of epigastric skin on the abdomen of the rat. The sandwich flap is then isolated with its supplying vasculature, transferred to the dorsum of the rat through a subcutaneous tunnel, and sutured in place. The inferior medio-lateral aspect of skin of the rat's abdomen is supplied and drained by the superficial epigastric vessels. Because of the reliability of these anatomical structures, this area has been identified as an area to study circulation in skin on a vascular island, i.e., skin supplied and drained by a defined vasculature (11). More recently, it has been used to develop and improve microsurgical techniques (12). An island skin flap is defined as a piece of living skin isolated and maintained by an independent and defined vasculature. Our sandwich flap is an island flap that has split-thickness skin grafted to its subcutaneous surface. The size of the flap is defined by the anatomy of the vascular bed (13), which can be readily visualized from the undersurface at the time of surgery. In this setting, the dermis of the donor skin and subcutaneous tissue of the host island flap grow together sandwiching the vessels supplying the flap, the superficial epigastric vessels.

Generation of skin sandwich flap

The island skin sandwich flap is constructed in three stages (Figure 1):

Stage I: Implantation of the STSG skin

Stage II: Lifting the flap from the rat abdomen

Stage III: Isolation of the flap vasculature and translocation to the rat back.

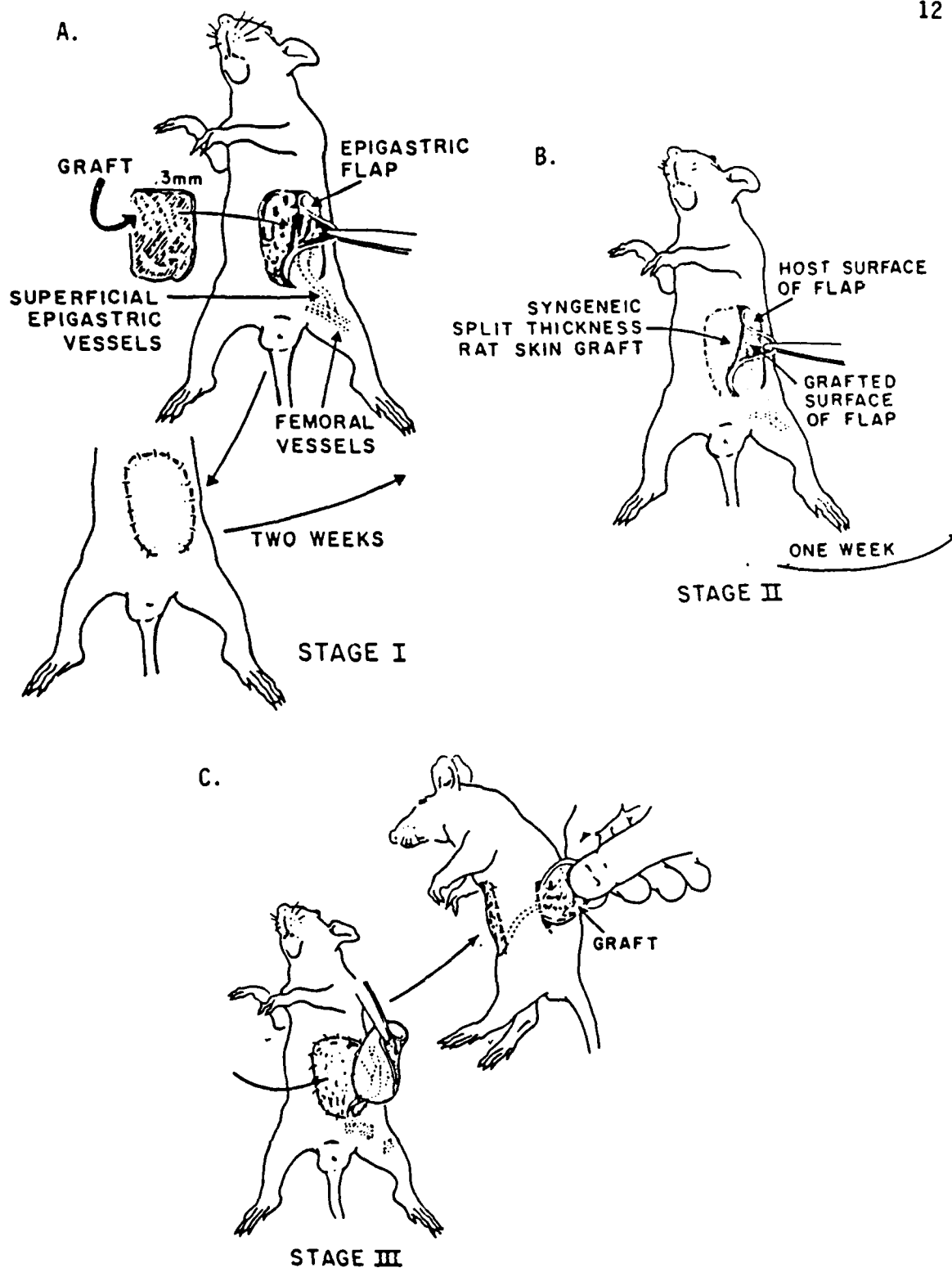


Figure 1. Schematic Diagram of Generation of a Rat-rat or Human-rat Skin Flap on the Congenitally Athymic (Nude) Rat. This diagram outlines the procedures for each of the stages utilized in generating the skin sandwich flap on an isolated but accessible vasculature.

For stage I, split-thickness skin grafts (STSG) are generated using syngeneic rat dorsal skin, or the human skin remnant from abdominoplasty procedure, which is dermatomed to a thickness of 0.5 mm. The STSG is either used immediately or stored in RPMI 1640 + 10% fetal calf serum at 4°C until used for grafting (not longer than 72 hours). The rat is anesthetized and immobilized on its back for the surgery. The sandwich flap is created by implanting a piece of STSG of syngeneic rat skin or human skin under the skin of the inferior lateral abdomen which is supplied and drained, for the greatest part, by the superficial epigastric vessels. The recipient area is generated by incising the abdominal skin of the rat along three sides and elevating this skin to its caudal base, at the level of the inguinal ligament. The STSG is trimmed to approximate the size of the host recipient area. The STSG is placed in the wound such that the epidermal side faces the abdominal musculature. The overlying epigastric flap is then returned to its normal anatomic position, where the flap of STSG and host abdominal skin are sutured in place (see Figure 1a). A bandage dressing of conforming gauze is applied to provide gentle pressure against the buried sandwich of skin as well as to inhibit the rat from scratching or gnawing at the surgical site. Our experience, as well as that of others, demonstrates that this surgical procedure does not lead to necrosis of the host skin, rather it generates a situation where the flap receives nearly all of its blood supply from the superficial epigastric vessel (11,14,15).

In stage II, the sandwich flap is isolated from the contiguous skin of the rat abdomen two weeks after stage I (see Figure 1b). The sandwich flap is freed from the adjoining abdominal host skin along the original suture line on three sides with a scissor, leaving the base of the flap intact. Histology (data not presented) reveals that vessels of the subcutaneous surface of the host flap grow into and establish a vascular network with the underlying STSG during this two week interval. The subsequent abdominal skin defect is fitted with syngeneic split-thickness rat skin (0.5 mm), epidermis side up, and sutured in place with interrupted stitches along the three sides and at the flap base with 5.0 monofilament nylon adhered to a PS-2 needle. Vaseline gauze is applied to both sides of the flap and the rat abdomen to maintain tissue hydration. A bandage dressing of conforming gauze is again applied and left in place for one week.

Twenty-one days after the initial surgery in stage I, the flap microcirculation has stabilized, as verified in a series of assessments with the laser Doppler velocimeter (data not presented). At this time, the vascular pedicle is created (Stage III) (see Figure 1c). Isolation of the superficial epigastric artery and vein as the primary source of blood supply and drainage is accomplished with careful dissection utilizing an operating microscope. The vessels proximal to the superficial epigastric vasculature, namely the pudendal artery and vein, are sacrificed by ligation and division. This manipulation increases the workable length of the femoral vessels so that the sandwich flap can be transferred to the rat's back. The femoral artery and vein distal to the origin of the superficial epigastric vessels are ligated individually proximally, together distally, and sacrificed. Bleeding sites are cauterized with a bipolar coagulator. These surgical manipulations maximize blood flow to the flap as all of the blood from the femoral artery which supplies the flap is shunted to the flap. The isolated skin sandwich flap with its attached vascular pedicle is translocated through a subcutaneous tunnel to the rat's back where it is sutured in place. The wound in the inguinal area is closed by mobilizing the surrounding skin into a primary closure. Collateral circulation

to the leg is sufficiently adequate to maintain the leg's viability and function. To inhibit the rat from chewing on the flap, the rat is restrictively bandaged from behind the forelegs to the front of the hindlegs with conforming gauze in such a way as to leave the flap free and exposed to air. Before the animal can be used experimentally, a period of two weeks is required to complete the healing following this final surgery. A typical skin sandwich flap in place and ready for experimentation is shown in Figure 2.

Accessibility of vasculature supplying skin flap

The re-isolation of the superficial epigastric vessels supplying the flap as they arise from the femoral vessel is accomplished with a simple incision over the inguinal ligament. The foregoing surgical procedures lead to some hypertrophy of the femoral artery and vein, thus making them more accessible. In a typical experiment, the opposite groin is exposed in a similar manner, the contralateral femoral vein being used for collection of systemic blood, the former site being used for collection of flap blood. These isolations are performed under the operating microscope at 18 X. To provide a degree of anticoagulation, heparin (10 IU/100 gm body weight in 0.7 ml of saline) is injected subcutaneously and intravenously immediately prior to experimental use. The venipuncture site to be utilized for the collection of experimental blood samples from the flap is made with a 30 gauge needle approximately 1 cm proximal to the origin of the femoral vein relative to the iliac vein. This 1 cm distance is chosen so that a microclamp can be placed on the femoral vein during blood collection to minimize possible reflux from the systemic circulation. Typically, blood samples (50 ul) are collected in heparinized microhematocrit tubes periodically throughout the experiment via capillary action when these tubes are placed over the venipuncture site. Hemostasis is produced by placing a cotton pledget over the site.

Dosage of cyclosporine to maintain human-rat flap

Initially, we reported that subcutaneous injection of 20 mg of cyclosporine on days 1-20 was all that was necessary to maintain the human skin in a viable state on the nude rat. Because rejections still occurred, we changed to a protocol which required the animals to receive 12.5 mg cyclosporine once per week for the duration of the flap life. The animals routinely lose 20-30% of their body weight during this treatment course. The loss of weight coincides with animal sickness which may, in fact, reflect cyclosporine toxicity. Poor animal health may also lead to flap chewing, a significant problem (see Results). In an attempt to alleviate some of the foregoing problems, the cyclosporine treatment protocol was further altered. Presently, animals receive 20 mg cyclosporine on day 1 and 12.5 mg subcutaneously every other day until day 21. At day 21, rats are placed on cyclosporine in sterile drinking water. The animals receive approximately 11 mg/kg/day when they consume what we calculate to be the average daily consumption of water, 30 ml/day. The cyclosporine water bottles are wrapped in aluminum foil due to the drug's photolability. The drinking water is held at a pH of 2.5 (see previous report), and in communication with Dr. L. Jacobs, Analytical Chemistry at Sandoz Laboratories, we have determined that this pH does not lead to degradation or decreased stability. The cyclosporine drinking water used in our studies is prepared from the commercially available cyclosporine for intravenous use. Water bottles are changed on a weekly basis. Currently, animals lose only 10% of their body weight through day 21; thereafter they either maintain or demonstrate an increase in body weight.



Figure 2. Photograph of a Rat-rat Skin Sandwich Flap on a Congenitally Athymic Nude Rat. This photograph was taken three weeks after stage III. The location of the flap is over the lateral abdomen, immediately anterior to the thigh of the nude rat. The surface depicted in this photograph is that of the rat host.

Percutaneous absorption using the skin sandwich flap

Figure 3 illustrates the model with host skin located on the top and the STSG located on the bottom of the flap. Investigational compounds can be applied to either the host or the graft side of the sandwich flap, either separately or simultaneously, the latter requiring different radiolabels or analytical methods which can detect nonradiolabelled compounds. In the experiments reported herein, the test compound is topically applied in a Teflon well (1.0 cm diameter). The LDV designation at the bottom of Figure 3 refers to laser Doppler velocimeter, and illustrates the site of attachment of this instrument.

Percutaneous application of benzoic acid

Benzoic acid has been used by a number of investigators to study percutaneous absorption (2,16-18). In our experiments with this agent, nude rats with a healed rat-rat skin sandwich flap are placed on a water heating pad to maintain an internal temperature of $37 \pm 0.5^\circ\text{C}$, the temperature being monitored with a rectal thermometer left in place throughout the entire experiment. Animals are anesthetized as described above and ^{14}C benzoic acid (specific activity 56.8 or 19.3 mCi/mM) in phosphate buffered saline vehicle is deposited onto the skin in the Teflon well attached to the flap skin (100 ul containing 220 ug ^{14}C -benzoic acid). This is immediately covered with Parafilm to minimize evaporation. The Parafilm is periodically lifted and 3 ul aliquots are collected and analyzed for changes in ^{14}C benzoic acid concentration in the donor well. Blood samples are collected as described above, centrifuged in a microhematocrit centrifuge, and plasma analyzed for DPM with liquid scintillation.

Percutaneous absorption of caffeine across human, rat host, and rat graft skin

^{14}C -caffeine (specific activity = 47.3 uCi/uM) dissolved in ethanol is deposited in 22 ug dosage in a Teflon well, 1.0 cm in diameter. The ethanol is allowed to evaporate and an assessment of percutaneous absorption is made as described for that of benzoic acid (see above).

Assessment of skin distribution of radioactive compound at the end of the collection of blood sample phase of an experiment.

The flap model system has some limitations for making a complete assessment of the amount absorbed per unit time. These limitations include the length of time the animal can be under anesthesia, the amount of blood that can be taken from an animal at any one time without causing hypovolemia and shock, and the fact that the investigators are unwilling to stay on the job site for more than 10 hours at a time. For these reasons, we elected to develop a method that localizes the distribution of the test compound in the skin at the end of an experiment. For this, a 2 mm punch biopsy is taken from the area of application of the test compound. This 2 mm biopsy is frozen in mounting medium and 50 micron sections are taken, by use of a cryostat, from one skin surface to the other skin surface (recall that the flap has two epidermal surfaces). Each of these 50 micron sections is solubilized, placed in a liquid scintillation cocktail, and the DPMs determined. From these data, a profile is made of distribution of the compound in the treated area of the flap. The total amount of radioactivity remaining in the treated site is calculated based on area, roughly 22 times that area contained in a 2 mm punch biopsy. For comparative purposes, a 2 mm punch biopsy is taken from another area of skin, typically from the back of the animal. Fixing the biopsy in formalin is not an

SCHEMATIC DIAGRAM OF A SANDWICH ISLAND FLAP

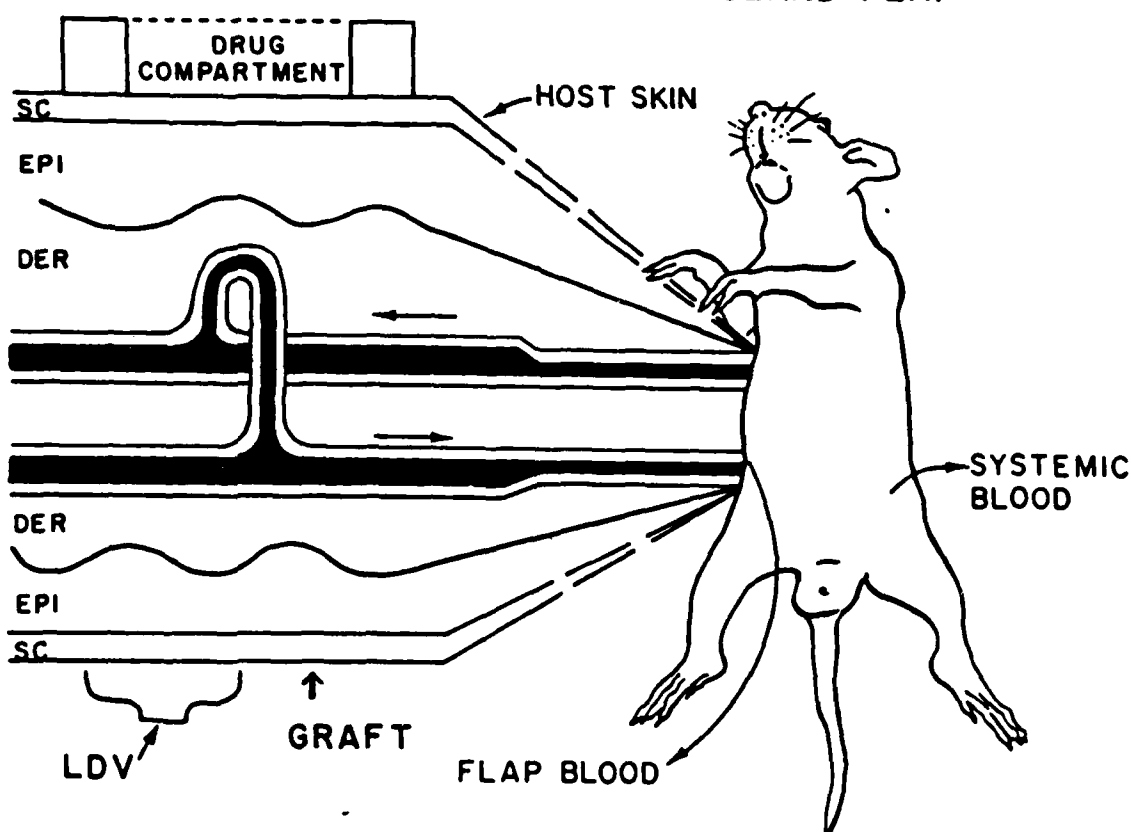


Figure 3. Schematic Diagram of a Sandwich Island Flap. Depicted in this diagram is the compartment where the test drug is applied and the position of the LDV relative to that compartment. Note that both the host and graft surfaces of the flap are served by a common vasculature. In a typical experiment, the systemic blood is drawn from femoral vessels of the groin opposite the origin of the vessels supplying the flap.

effective way of assessing distribution, as compounds that are not covalently linked to skin or a receptor will freely diffuse from the skin. Assessment of radioactivity of the mounting media, as well as the tin foil in which the specimen is placed, reveals the foregoing technique to result in minimal leeching of radioactivity from the specimen.

Theoretical considerations and calculations

For the purposes of continuity and maximal relation to the literature, the data are presented as accumulated counts as a percentage of the applied dose, or as nanograms in the case of tissue localization experiments (see above). The percent of original dose is calculated as the area under the curve (AUC) by the trapezoidal rule as a concentration time curve ($\mu\text{g}/\mu\text{l} \times \text{min}$ vs. time). Because we demonstrate (see Results) that blood flow is critical to the amount of a compound that is absorbed over time, data are corrected for blood flow, $[(\mu\text{l}/\text{min})(\mu\text{g}/\mu\text{l} \times \text{min})]$.

Another useful way of demonstrating the data is as flux. The flux of the study compound that enters the systemic circulation after transdermal absorption across the skin flap (J_i) is given by the equation: $J_i = (C_f - C_b)Q_f/A$ where C_f is the concentration of the compound in the venous blood exiting the skin sandwich flap, C_b is the concentration of the compound in the recirculating blood, i.e. the systemic blood, entering the flap, Q_f is the rate of blood flow through the flap, and A is the skin surface area. Area under the curve (AUC) is calculated by the trapezoidal rule as a concentration-time curve ($\mu\text{g}/\mu\text{l} \times \text{min}$ vs time). When the concentration-time curve is corrected for blood flow $[(\mu\text{l}/\text{min})(\mu\text{g}/\mu\text{l} \times \text{min})]$, the data are plotted as μg drug versus time.

Orthotopic transplantation of split-thickness scalp grafts

These experiments were designed to determine whether or not human scalp skin could be grafted to the nude rat, whether or not various thicknesses determined density of subsequent hair growth, and finally whether or not cyclosporine affected hair growth. The human skin utilized in these experiments consisted of remnants of scalp skin removed during elective "face-lift" surgery. These remnants were trimmed to the appropriate thickness (0.4 mm or 1.0 mm) with a dermatome (Brown Electro-Dermatome, model 666, Zimmer USA, Warsaw IN). Prior to trimming the scalp skin remnants, the existing hair was trimmed to the skin surface by shaving with a safety razor. Prior to grafting, the rats were anesthetized with ketamine hydrochloride, 1 mg/10 gm body weight (Ketalar, Parke-Davis, Morris Plains NJ). The skin overlying the recipient site, the lateral thorax, was surgically excised to the depth of the panniculus carnosus to a size appropriate for the HSTSG of scalp skin (HSTSG-SS). Bleeding was controlled with pressure. The HSTSG-SS was cut to the desired size (1-4 cm in diameter) with a sterile cork borer, placed on the recipient site, and sutured in place with 5-0 silk sutures. The graft sites were covered with a vaseline impregnated gauze and further secured by wrapping the thoracic cage with several layers of surgical tape. The dressings and sutures were removed on day 10; thereafter, the grafts were inspected daily.

Cyclosporine (CS) (50 mg/ml, 5 ml vial, Sandoz Laboratories, East Hanover NJ) was injected subcutaneously, 25 $\mu\text{g}/\text{kg}/\text{day}$, for 10 weeks to 10 rats grafted with 0.4 mm thick HSTSG-SS and to five rats grafted with 1 mm thick HSTSG-SS. Four rats were grafted with 0.4 mm thick HSTSG-SS and treated with other immunosuppressive agents. In this group, two rats were given intravenous

injections of 0.5 cc undiluted anti-lymphocyte serum (ALS) (kindly supplied by Charles W. DeWitt, Ph.D., Department of Pathology, University of Utah School of Medicine, Salt Lake City UT). These rats received injections on four consecutive days and then on a weekly basis for a total of seven weeks. Within nine days of discontinuing the ALS, the grafts were rejected. Two grafted rats received other treatments: one was treated with azathioprine (Imuran, Burroughs Wellcome Co., Research Triangle Park NC), 5 mg/kg/day intraperitoneally and the other received intraperitoneal injections of azathioprine (at above dosage) and hydrocortisone succinate, 0.5 cc (Solu-Cortef, Upjohn Company, Kalamazoo MI), on a daily basis for five weeks. Without immunosuppressive therapy, rejection of HSTSG-SS occurred in 29+9 days.

Biopsies were taken from the HSTSG-SS before and at intervals following grafting. Fixation of tissue for routine histologic sections was carried out in 10% neutral buffered formaldehyde solution. Horizontal or vertical sections were made of these biopsies, stained with hematoxylin and eosin, and examined under light microscopy.

As an additional method of assessing hair growth, the number of hairs per graft and the hair length were determined under 5x magnification. Hair shaft diameter was measured on transverse sections with the use of a calibrated ocular micrometer (19).

Results/Discussion

Blood flow in the flap

We have previously demonstrated (see second annual report) that blood flow to the flap is critical to the clearance of a percutaneously absorbed compound. In that assessment, there was a direct correlation between blood flow, as determined by laser Doppler velocimetry, and the quantity of benzoic acid absorbed.

This observation led to the following questions: 1) Do LDV values correlate with actual blood flow, i.e., ml/min of blood supplying the flap? 2) If there is a correlation, can that be confirmed by a second methodology? 3) Does blood flow to the flap change as a result of flap aging? 4) Does blood flow differ when the graft side of the flap is compared with the host side? and 5) Does cyclosporine affect the microcirculation of the skin?

Correlation of actual blood flow with LDV and confirmation of LDV with dermal fluorometry

Presently, the laser Doppler velocimeter (LDV) is used to non-invasively monitor flap blood flow throughout an experiment. The analogue read-out of the LDV is in millivolts. This is an awkward unit for describing volume of blood per time. For this reason, it became desirable to correlate the LDV millivolt units to actual milliliters of blood flow per minute. A new instrument, the electromagnetic blood flow meter (EBFM) utilizes a "C-type probe" which is attached directly to the superficial epigastric artery supplying the flap. This instrument, although invasive, directly evaluates blood flow and has been used to correlate LDV values of skin with actual blood flow rates (Note: This type of direct correlation, actual blood supply vs. millivolts as measured by the LDV, has never been made). In this experiment we utilized a nude rat (W1) weighing 179 gms on cyclosporine drinking water, with a healthy rat-human skin sandwich flap. Blood flow was manipulated by altering the temperature of the

animal and by mechanical pressure proximal to the origin of the superficial epigastric artery. Using these maneuvers, whenever the EBFM had a reading of 0, 1, 2, or 3 ml/min, the corresponding LDV value was recorded. The LDV was applied to the human graft component of the flap in the usual manner (see Methods). The number of LDV readings was: 36 at 0 ml/min, 124 at 1 ml/min, 261 at 2 ml/min, and 44 at 3 ml/min. As demonstrated in Figure 4, there is a highly significant correlation between LDV values and actual blood flow to the flap. Previously, we have demonstrated that the correlation between arterial blood flow and venous blood flow to and from the flap is absolute (data not presented). The value of 55 mv without evidence of blood flow is a value that is routinely seen, e.g., attaching the LDV to nonviable human skin prior to grafting will typically result in LDV values ranging from 40 to 60.

A similar assessment (data not presented) has been made at the end of stage II. In that assessment, the slope of the correlation is not as steep, 13.5 mv/ml/min vs. 37.7 mv/ml/min. This analysis confirms our contention that the microvascular surgery at stage III, surgery to ensure a sole source of blood supply to and from the flap, does indeed reduce the collateral circulation to the flap by approximately 25 mv.

The appearance and disappearance of fluorescein from the skin, following an IV injection, directly correlates with capillary blood flow and renal clearance (see second annual report). The appearance and disappearance of fluorescein from the skin can be measured in a non-invasive manner with a surface dermal fluorometer (DF). We wished to confirm the notion that LDV measurements do provide a direct assessment of blood flow to the skin. Accordingly, an analysis of the ratio of LDV values to DF values has been undertaken, the results of which are presented in Figure 5. Blood flow was altered in these experiments by delivering phenylephrine, iontophoretically, to a selected site on the flap. This experiment has been repeated on four different flaps as a percent of relative index (see Figure 5). The relative index was calculated by determining the experimental dermal fluorometer value in the vasoconstricted area with that of control area. The percent relative index for the LDV was calculated in a similar manner. This correlative analysis demonstrates a high degree of correlation, $r = 0.95$, and provides confirmation that the LDV is measuring blood flow. Knowing the weight of an average flap (10 gms) and the blood flow to the flap allows us to calculate blood flow volume per time per weight. While these types of values are not important at this time, they will be important in the future when perfusion becomes an important issue. At this time, it appears that the average blood flow to the flap is approximately 20 ml/min/100 gms.

Comparative analysis of blood flow to the flap as a function of age, graft vs. host, and effect of cyclosporine.

It is recognized that following skin injury healing processes go on for a considerable period of time. Because of this, we are obviously concerned about blood flow as a function of age of the flap and of blood flow of the graft vs. the host side of the flap.

In our experimental system, the LDV is placed on the flap and left on throughout the entire experiment. At the end of the experiment, an average LDV value is obtained. Extrapolating LDV values to ml/min (see Figure 4) provides blood flow as ml/min. These values are plotted as a function of flap age (see Figure 6). Flap age is determined from the end of stage III. As can be noted,

CORRELATION OF LDV VALUES WITH
MEASURED BLOOD FLOW TO THE FLAP

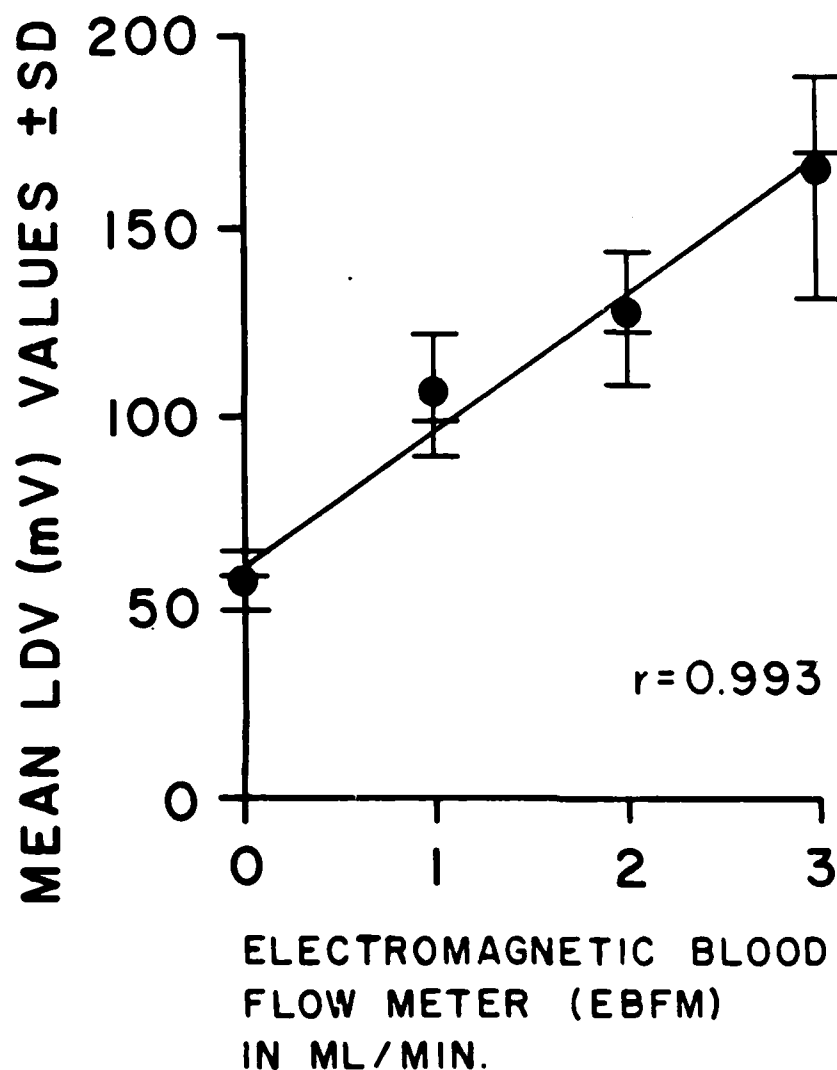


Figure 4. Correlation of LDV Values with Measured Blood Flow to the Flap. In this experiment, blood flow of the artery supplying the flap was measured with an electromagnetic blood flow meter, ml/min. When these values were 0, 1, 2, or 3, LDV values were recorded. These numbers were plotted, revealing a highly significant correlation between LDV values and actual blood flow.

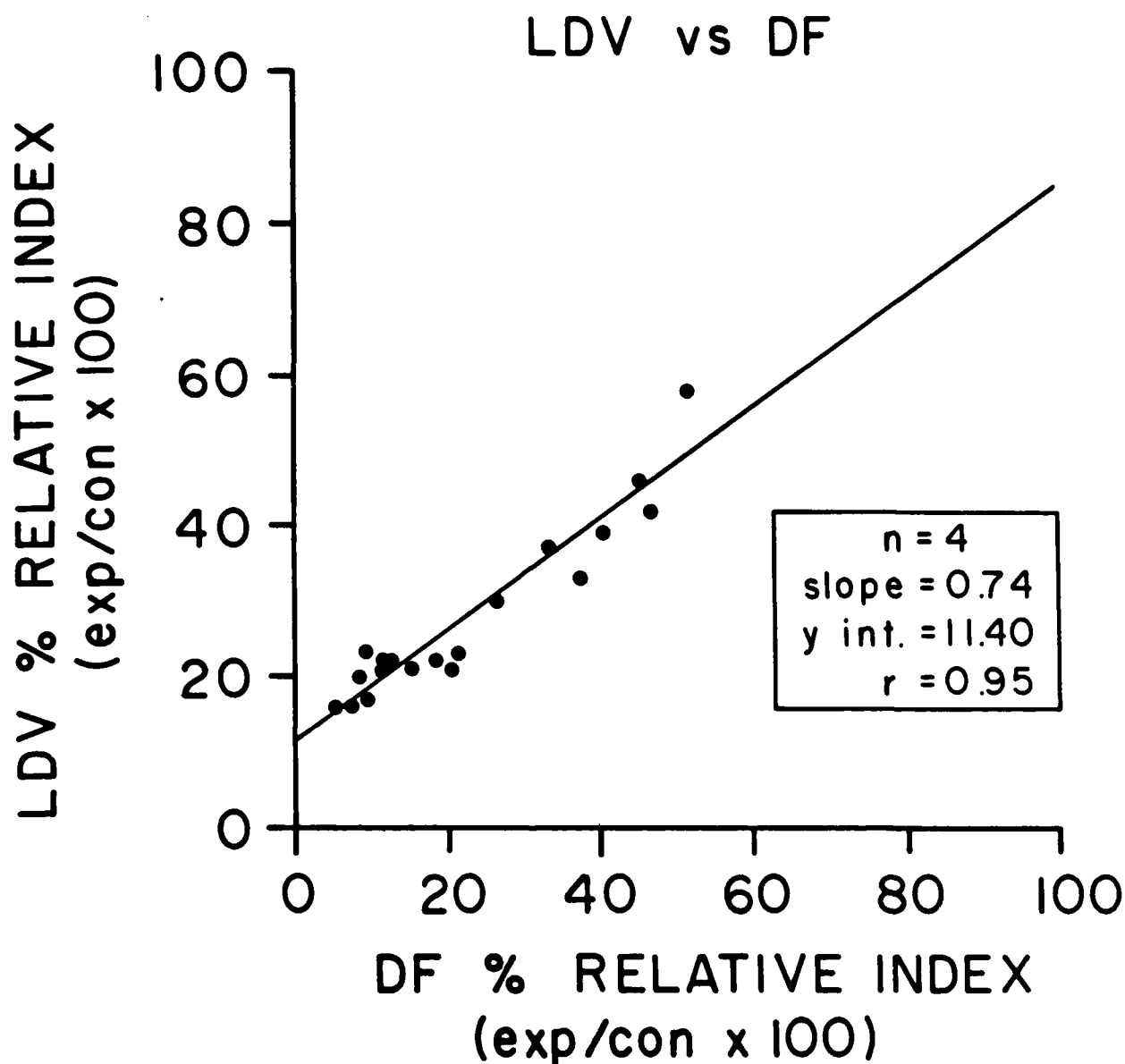


Figure 5. Correlation of LDV Values with Clearance of Fluorescein From the Skin as Measured with the Dermal Fluorometer (DF). Vasoconstriction was accomplished with delivery of phenylephrine via iontophoresis, and the relative index was calculated by comparing instrument reading values in the vasoconstricted area with that immediately adjacent to the vasoconstricted area. A highly significant correlation is present.

the number of flaps at any particular time period decreases as a function of flap age, seven at week 2, to one at week 12 and thereafter. Curiously, the animal at week 12 and thereafter has the same inherent variability as seen in individual animals prior to week 12. These data suggest that the inherent variability that is seen is not a function of age. Further, while the average blood flow is near 2 ml/min, there are experiments where the average blood flow was in excess of 5 ml/min (see week 6 data in Figure 6).

To assess the blood flow of the host side of the flap with that of the graft side, an analysis of the LDV values of the graft and host sides have been made as a function of flap age (Figure 7). In these numerous examples, $n = 30$, there is no change in the blood flow ratio, graft vs. host, as the flap ages. It is generally noted that the graft side of the flap has less blood flow, mean blood flow ratio graft/host = .80 at the end of stage III (0 weeks), to .76 at the end of 24 weeks. However, there are eight cases (27%) where blood flow on the graft side was greater than that on the host. A correlation coefficient demonstrates no significance, $r = -0.05$. These data suggest that blood flow does not significantly change in either the graft or the host side as the flap ages. Further, the variability seen in Figure 7 is not solely dependent upon either the graft or the host side. Similarly, these differences appear unrelated to temperature, as body temperature was monitored throughout many experiments and there is no correlation with cutaneous blood flow. Temperature-dependent blood flow changes require greater shifts in temperature than we customarily encountered (0.5°C).

To address the question of the effect of cyclosporine, rat-rat flaps have been generated in cyclosporine and non-cyclosporine treated animals. As can be noted in Table 1, the ratio of blood flow in the presence and absence of cyclosporine is unchanged. The overall mean = $.81 \pm .26$. The values in the presence and absence of cyclosporine are not significantly different from this. The same assessment is true for the human-rat flaps.

Table 1
Blood Flow Ratios^a in the Skin Sandwich Flap

Flap Type	+CS RX	-CS RX
Rat/Rat	$0.85^b \pm 0.26$ (7) ^c	0.80 ± 0.25 (18)
Human/Rat	0.78 ± 0.23 (12)	Not available

^a graft LDV value/host LDV value

^b mean \pm SD

^c number of animals

+CS RX = treatment with cyclosporine

-CS RS = no treatment with cyclosporine

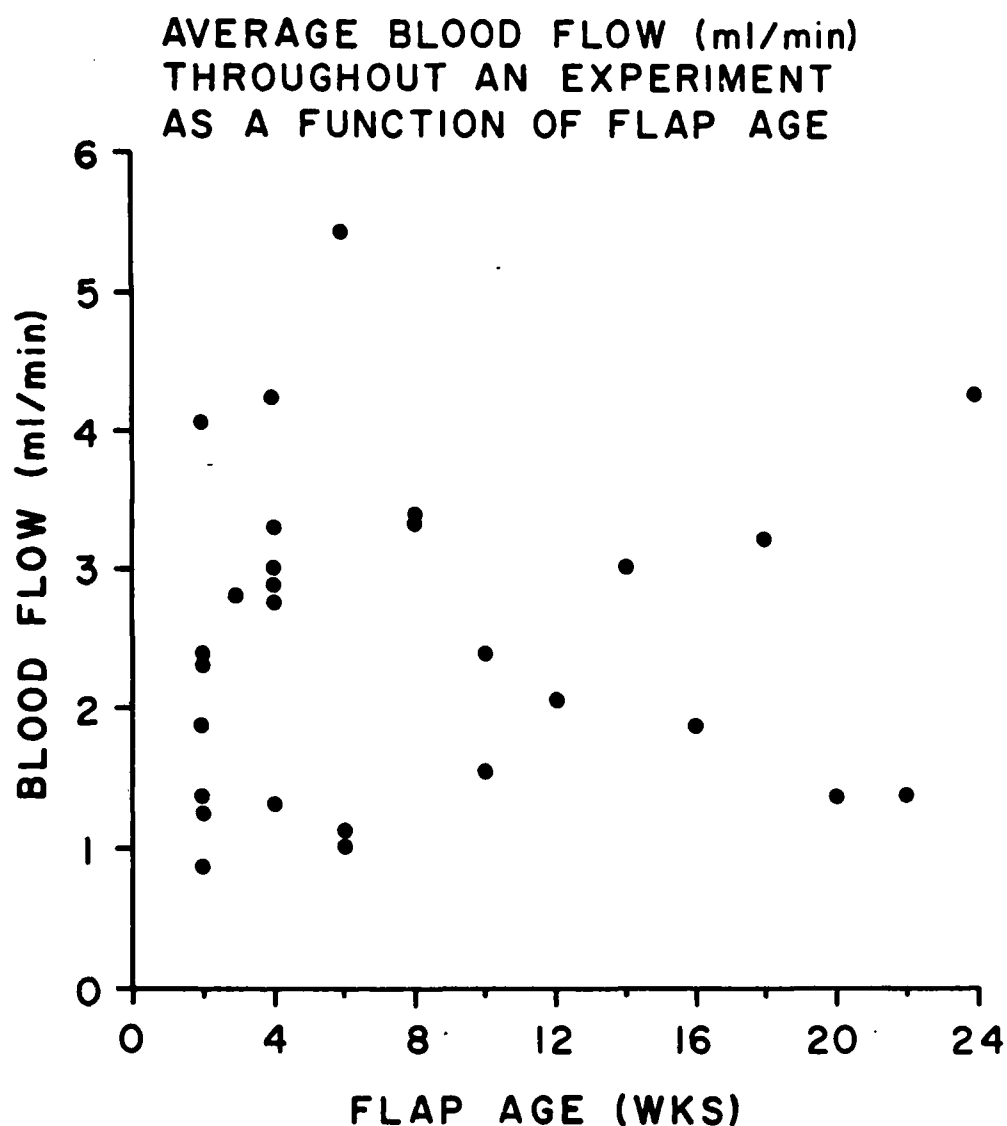


Figure 6. Average Blood Flow as a Function of Flap Age. Average blood flow is illustrated as ml/min, calculated from the slope of values presented in Figure 5 during an experiment, as a function of flap age. Each point represents an individual flap at that particular flap age. After week 12, each point represents the same flap (Z1) at 2 week intervals. Note the high rate of blood flow in the old flap, age 24 weeks.

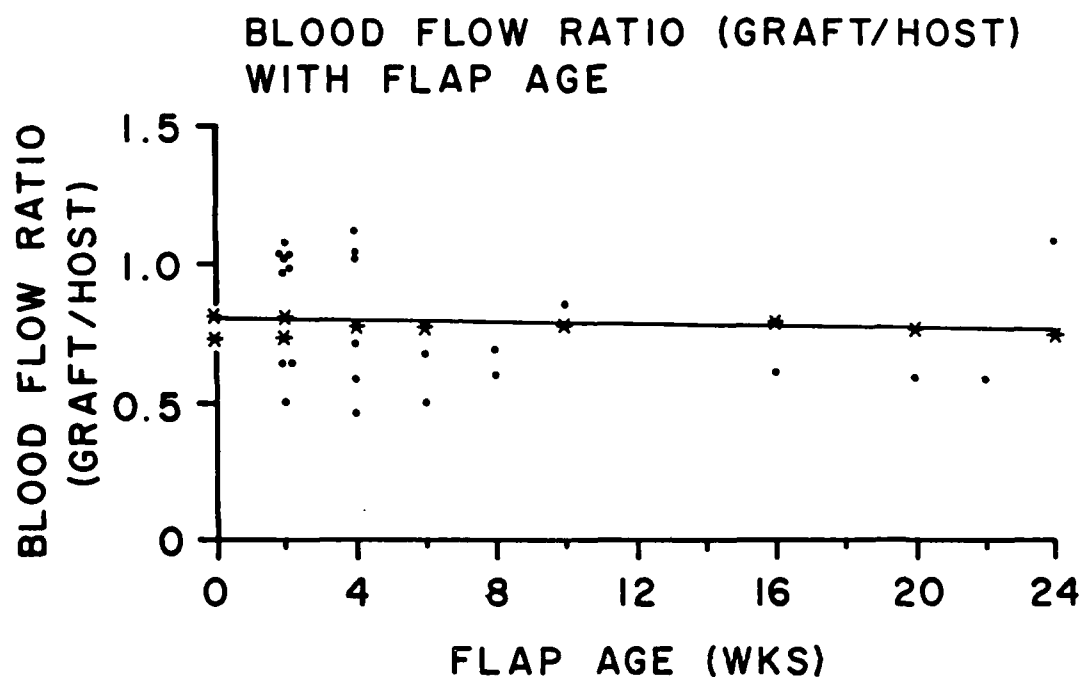


Figure 7. Blood Flow of the Graft Side vs. the Host Side of the Flap. Blood flow was compared as a ratio with age of the flap. Mean blood flow is not significantly altered as a function of age when a correlation coefficient is made, $r=-0.05$. Generally, the host side of the flap has greater blood flow, as measured with the LDV, than does the graft side.

Influence of blood flow on percutaneous absorption of benzoic acid

Previously, we reported (see second annual report) that altering blood flow alters percutaneous absorption of benzoic acid across the rat graft component of a rat-rat skin flap. This analysis has been extended to address the question of whether the biologic variability in the blood flow alters the amount of benzoic acid absorbed, and then to also determine how blood flow affects flux.

A series of experiments was developed to determine the influence of flap age on amount of benzoic acid that is absorbed. These experiments are illustrated in Figure 8A&B. As noted in Figure 8A, there is considerable difference in absorption and there is no correlation of this absorption with the age of the flap. Because previous experience suggested that blood flow was critical to the absorption of benzoic acid, and because blood flow is highly variable (see Figures 6 and 7), the absorption was corrected for actual blood flow (see Methods). These data are presented in Figure 8B. As can be appreciated, correcting for blood flow causes the data to become very similar, i.e., percent of original dose at 4 hours being very similar regardless of age. This analysis again confirms the necessity of understanding blood flow to the skin when attempting to accurately determine the amount of a compound that is absorbed in the early time points following topical application. The early time points are especially critical in situations where toxic compounds are delivered topically.

Preliminary experiments have also suggested that phenylephrine, 1 mM in distilled water, iontophoretically delivered to the skin, 0.5 mA for 15 minutes, will effect a local vasoconstriction that lasts approximately 30 minutes. A comparison of blood flow, as measured by the LDV, has not been made on skin that has undergone control treatment, iontophoresis with water. This experiment is presented in Figure 9. Following iontophoresis of phenylephrine, there is a rapid decrease in microcirculation to the skin, as measured by the LDV, which begins to recover within 20-30 minutes and normalizes by 2-3 hours. Not displayed in this experiment is the frequent observation of rebound, i.e., blood flow after recovery being higher than that at baseline. The iontophoresis of water, and the no iontophoresis components of this experiment demonstrate a slight increase in blood flow as a function of time. This increase is not unusual, but is not sufficient to reach significance. The same flap was used for each of these experiments, the experiments being conducted at two week intervals. The difference in baseline again represents the variability previously addressed.

Figure 10 demonstrates the flux of benzoic acid across the grafted surface of a rat-rat skin flap following iontophoresis of water or the iontophoresis of phenylephrine. These fluxes are compared with those of benzoic acid without iontophoresis. In these analyses, it is apparent that iontophoresis of water or phenylephrine changes the barrier function such that greater amounts of benzoic acid are absorbed. In comparing flux as a function of time in these experiments, it is noted that phenylephrine alters absorption in a major way. The values represent mean values of duplicate experiments. In this vasoconstricted scenario, it appears that benzoic acid moves through the stratum corneum and the epidermis and remains in the dermis until microcirculation is restored. Thereafter, a rapid flux of benzoic acid into the bloodstream is noted. In these experiments, there was reflux hyperemia at 2 and 3 hours. It is proposed that the hyperemia is the reason for the flux dropping to zero at the time the experiments were concluded. The nearly

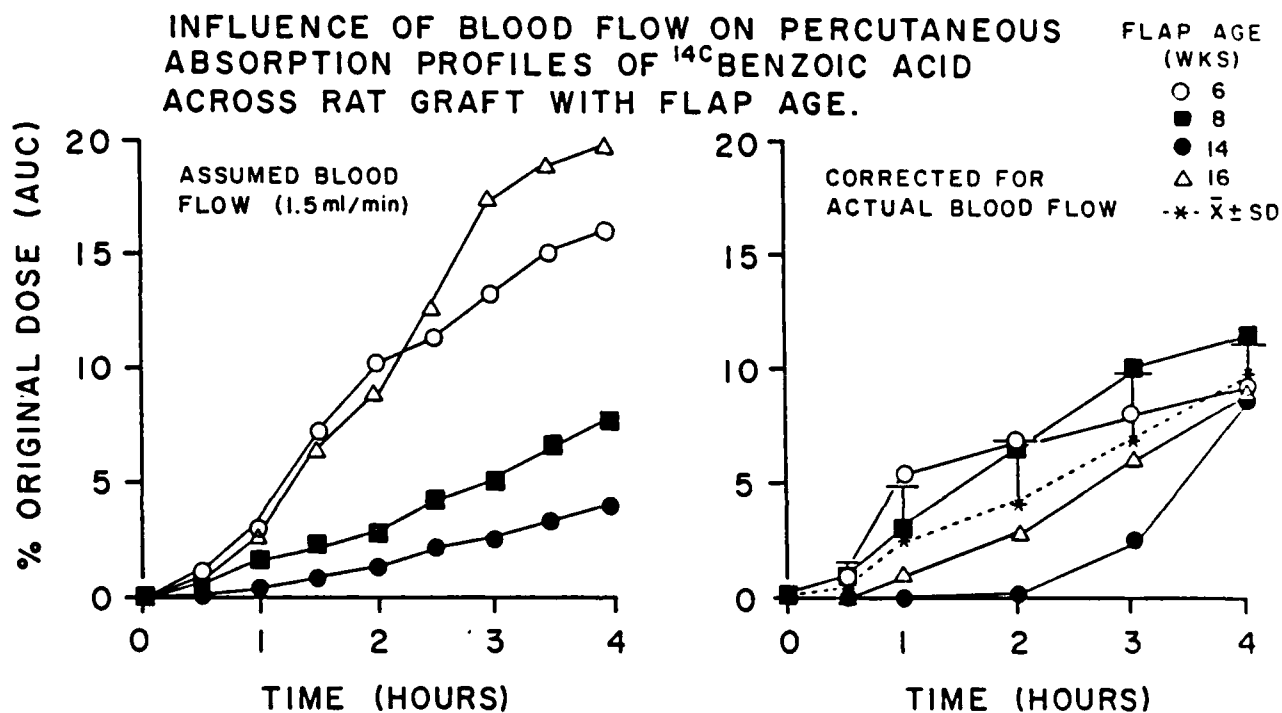


Figure 8. Influence of Blood Flow on Percutaneous Absorption Profiles of ^{14}C -Benzoic Acid Across the Rat Graft, as a Function of Age. Data are presented as a percent of the original dose absorbed. This experiment was performed on the same animal at an average of 2 week intervals (see above). The left side of the figure represents Figure 8A and the right side represents Figure 8B (see text). The lower half of the standard deviation bar is not included for mean percent absorption of original dose.

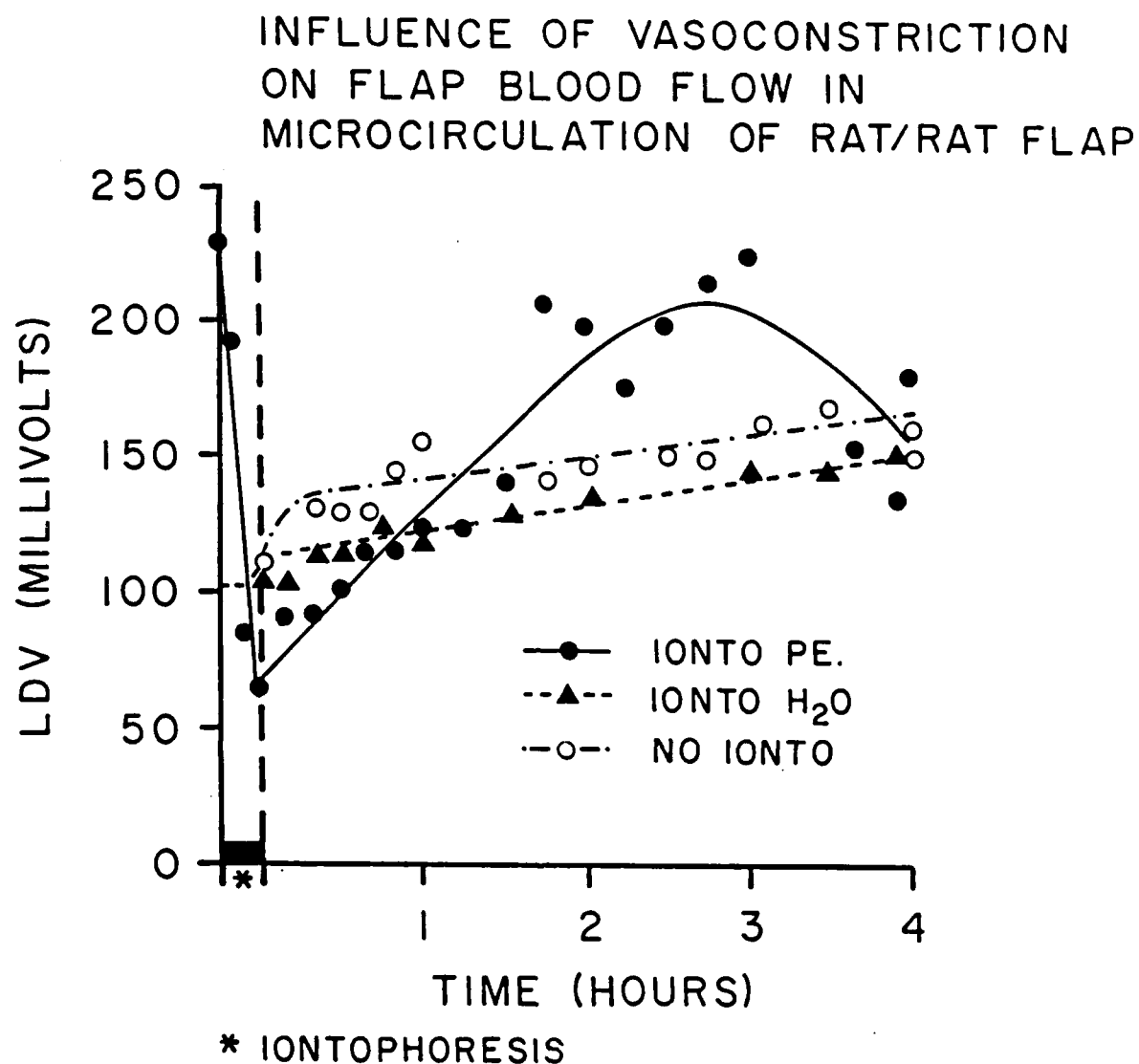


Figure 9. Influence of Vasoconstriction on Flap Blood Flow in Microcirculation of the Rat-rat Flap. Plotted values represent LDV readings after iontophoresis of 1 mM phenylephrine, 15 min at 0.5 mA. These values are compared with that of iontophoresis of water alone and both are compared to no iontophoresis. Each point represents a LDV reading at that time point.

EFFECTS OF IONTOPHORESIS ON PERCUTANEOUS ABSORPTION OF ^{14}C BENZOIC ACID (BA) $283\mu\text{g}/\text{cm}^2$

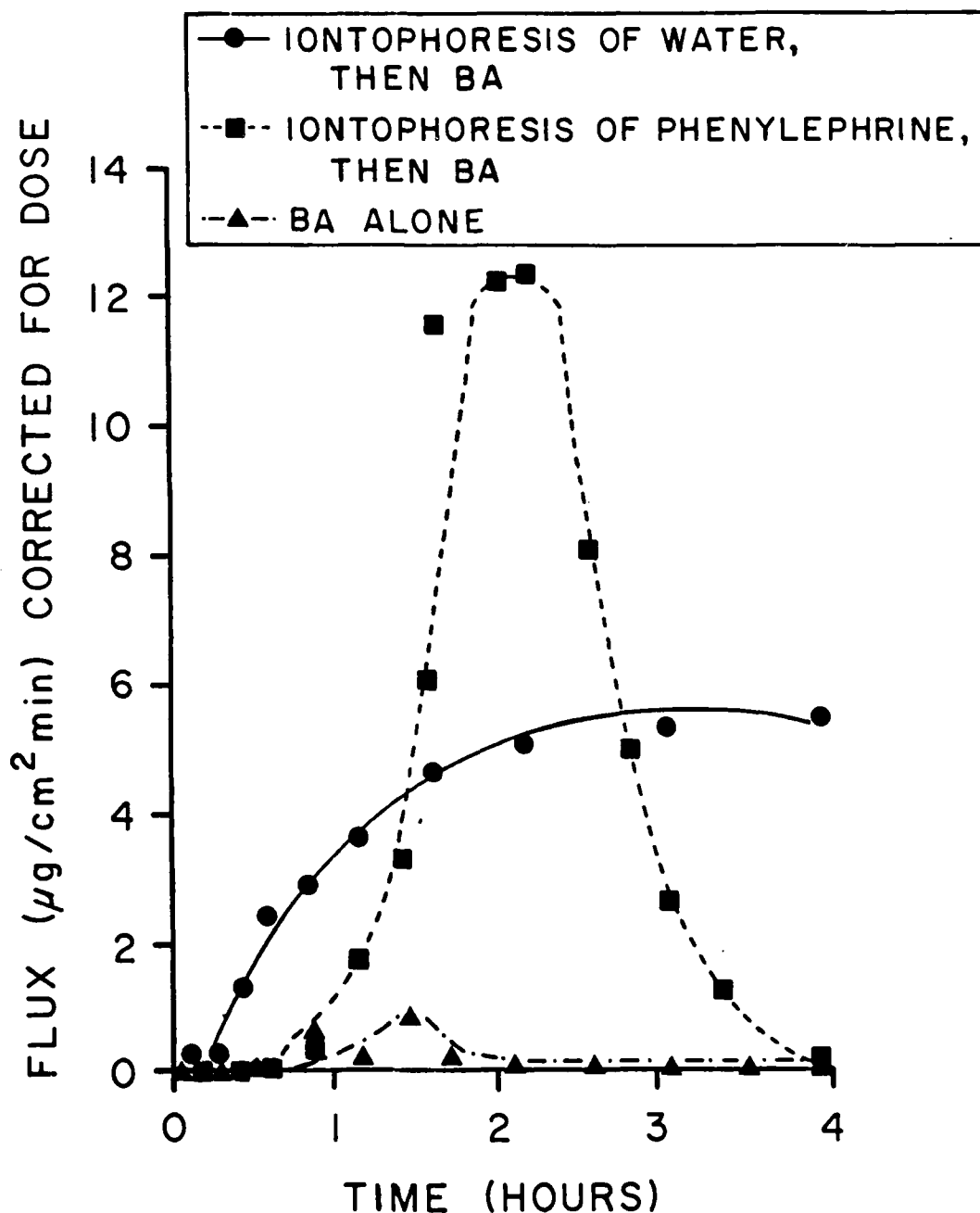


Figure 10. Effects of Iontophoresis on Percutaneous Absorption of ^{14}C -Benzoic Acid. Values represent the mean of two experiments. Each experiment was performed on the same animal at 2 week intervals, i.e., benzoic acid in buffered vehicle alone, 2 weeks later repeat of the experiment. Prior to the application of benzoic acid, iontophoresis of water or phenylephrine was carried out (see Figure 9). Data are presented as flux corrected for the dose applied.

infinite dose of benzoic acid on the surface should have been sufficient to maintain a steady state appearance such as seen with iontophoresis of water alone (see Figure 10). Had these experiments been conducted for longer periods of time, it is likely that detectable amounts of benzoic acid would have been seen, once again, in the flap blood of the flap treated with phenyephrine. These experiments confirm our previous suspicion that blood flow is an important parameter when assessing percutaneous absorption and that alterations in the stratum corneum caused by hydration, i.e., iontophoresis of water alone, also substantively change absorption.

Comparison of absorption of caffeine, rat skin vs. human skin, on the rat skin flap, and in vivo vs. in vitro using human skin

These studies employed the use of ^{14}C -caffeine in an ethanogl vehicle deposited on the skin in a Teflon ring, dosage applied 36 ug/cm^2 , and were designed to assess the critical questions of differences in absorption across grafted and nongrafted surfaces, across human and rat skin, and across human skin in vitro vs. in vivo.

Comparison of absorption of caffeine, rat host, rat graft, and human graft on the rat skin flap system

These experiments were designed to quantify differences in these three different types of skin. The proposed hypothesis is that if the profile of absorption across rat graft and rat host do not differ significantly, it follows that absorption across human skin graft on the flap and human skin in the in situ in vivo state (on the host) would also be very similar. As Figure 11 demonstrates, the difference in percent of original dose absorbed as a function of time, rat host vs. rat graft, is virtually the same. These values, means of three experiments through two hours, demonstrate that much more caffeine is absorbed across rat skin than across human skin. By the end of four hours, the amount having crossed human skin is nearly 10 times less than that which has crossed the rat skin. These data suggest that although the nude rat is, for the most part, hairless, the absorption profile of caffeine across this type of skin is quite different from across human skin. Further, there is no essential difference between the absorption profile of rat graft skin and that of rat host skin. These data serve to validate the system and cause us to predict that the absorption of compounds across human skin in situ will be very similar to that seen across the human component of our skin flap system.

Percutaneous absorption in vivo vs. in vitro

Human skin, 0.5 mm in thickness, was applied to a Franz cell and the same dosage of caffeine, corrected for area, was applied. Skin from these same donors was used to generate flaps on nude rats. These resultant flaps were then subjected to the same dosage of caffeine. This experiment was repeated in triplicate (results presented in Figure 12). This analysis demonstrates a significantly higher amount of caffeine being absorbed through the skin in the in vivo setting when compared with that of the in vitro setting. This enhanced absorption in vivo was unexpected, as we anticipated that viable, functional skin would have better barrier properties than nonviable skin.

Binding of caffeine in vivo vs. in vitro

The foregoing suggested the obvious, namely that caffeine that is absorbed in the in vivo state has only to cross to the capillary bed where it is absorbed, whereas caffeine that is absorbed in vitro has to traverse the entire dermis before appearing in the receiver chamber. To address this question, an

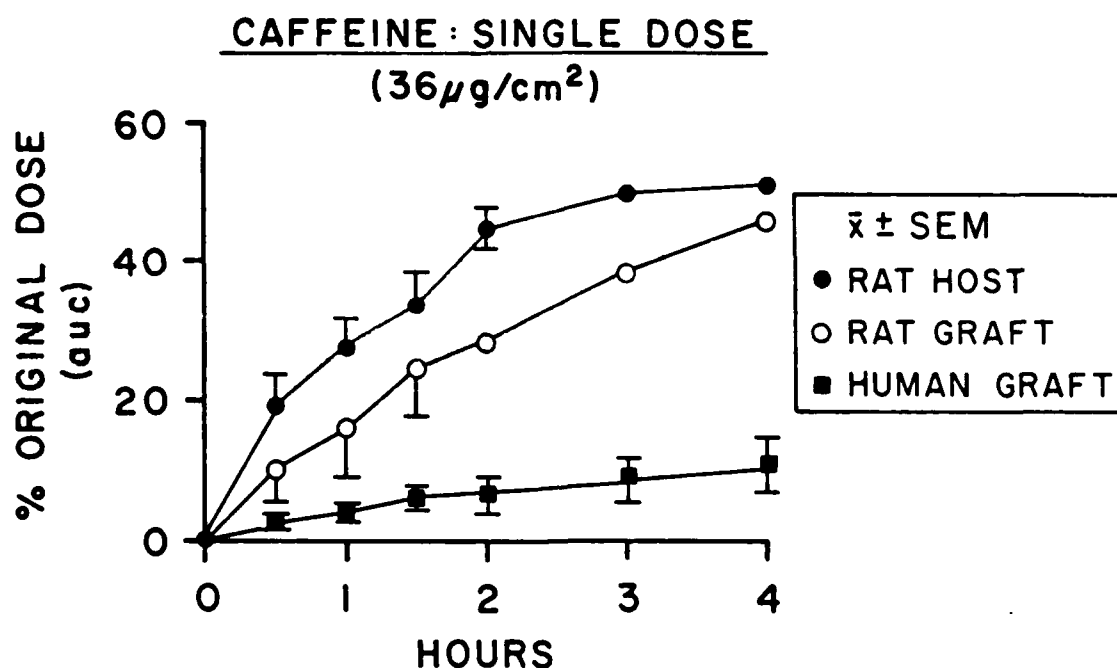


Figure 11. Comparison of the Absorption of Caffeine. Comparison is made of absorption of caffeine across rat host, rat graft, and human graft of the rat flap system. Experiments for the human graft component show mean values of three experiments on three different flaps. Values for rat host and rat graft are from two different flaps. Values at 2, 3, and 4 hours are data from a single rat flap, either host or graft.

PERCUTANEOUS ABSORPTION OF SINGLE
DOSE ^{14}C CAFFEINE ($36\mu\text{g}/\text{cm}^2$) ACROSS
SPLIT/THICKNESS HUMAN SKIN:
IN VIVO VS. IN VITRO

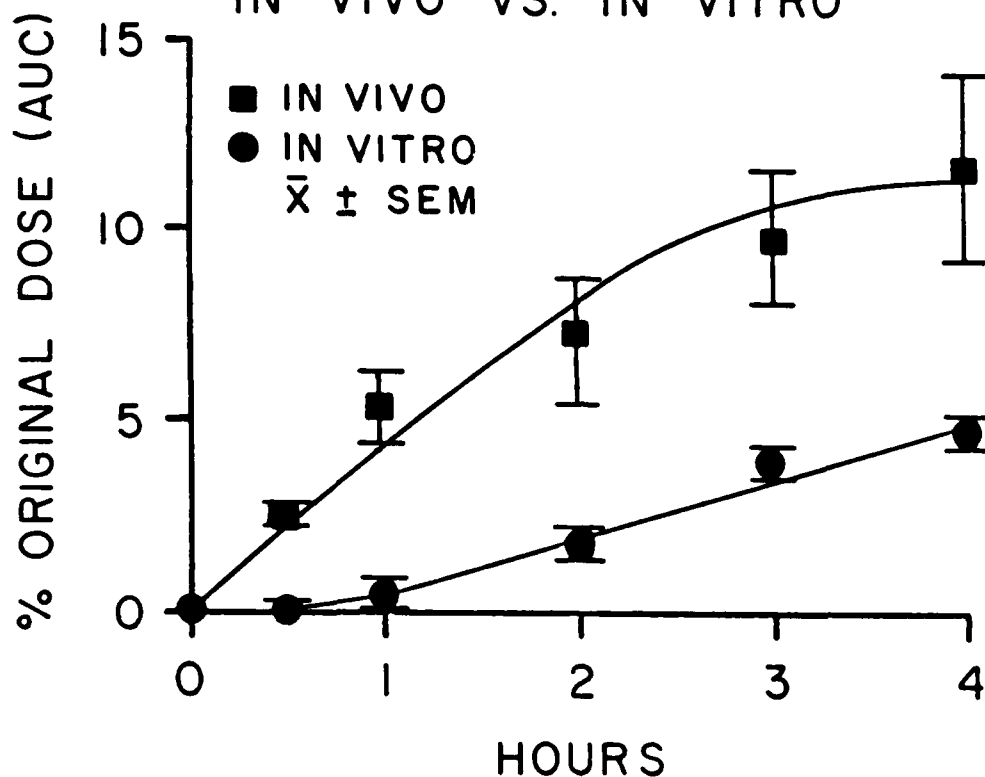


Figure 12. Percutaneous Absorption of a Single Dose of ^{14}C -Caffeine. Comparison is made of percutaneous absorption of ^{14}C -caffeine across a split-thickness human skin graft, in vivo vs. in vitro. Values represent the mean of triplicate experiments, each performed with a different skin source. However, the in vivo skin source and the in vitro skin source for any one experiment are from the same donor (the in vitro experiments were done prior to the in vivo experiments). Data are presented as percent of the original dose absorbed, area under the curve (AUC).

additional experiment has been performed, again in triplicate (see Figure 13). In this experiment, a second dose of caffeine was added in vivo at a time when steady state was developing (just prior to 2 hours). Similarly, a second dose was applied in vitro at 3 hours. In this experiment, the deposition of a single dose of caffeine (36 ug/cm^2) results in approximately 5% of the original dose being absorbed by 4 hours. The total amount of caffeine absorbed after two 36 ug dosages of caffeine results in an absorption of approximately 96% of the second dose. This is 19-fold greater than that expected by simple linear absorption. The addition of a second dose in vitro demonstrates no enhanced absorption of the second dose. Further, it is noted that the total amount of caffeine absorbed in vitro is significantly less than that in vivo. These data suggest tissue binding (see below) and demonstrate the importance of basing conclusions relative to percutaneous absorption on in vivo analyses.

Tissue binding, rat-rat and human-rat skin sandwich flaps following the deposition of 36 ug/cm^2 of ^{14}C -caffeine

The foregoing suggested that caffeine binds to the dermis both in vivo and in vitro. Because there is less dermis between the absorption site (stratum corneum) and the capillaries, caffeine will appear more quickly in the capillaries in the in vivo state than in the in vitro state where caffeine must traverse the entire dermis. To address this possibility, biopsies have been taken of rat-rat and human-rat flaps following deposition of ^{14}C -caffeine. These 2 mm biopsies are then sectioned horizontally from surface stratum corneum to surface stratum corneum at 50 micron intervals. The radioactivity in each 50 micron skin section is determined with liquid scintillation counting, and the profile of distribution is plotted in Figure 14. This analysis reveals significant binding in the mid-dermis, 400-800 μm in human skin. Thereafter, there is a linear decrease until the opposite side of the flap, rat skin, is reached. Note that the vertical axis is presented as nanograms while the total amount absorbed in the blood is in the microgram range (see below). The distribution profile following percutaneous application on a rat-rat flap demonstrates the grafted side to also have significant counts in the mid-dermis, 200-600 μm ; the opposite host side showed significantly less caffeine in this experiment. The rat host side of the human flap demonstrated a significant amount of binding, again in the dermis. This is interpreted as evidence of binding of caffeine via the systemic circulation and has been confirmed by a similar profile following the same type of analysis of back skin of a rat after completion of an experiment (data not presented). The decreased amount in the rat-rat flap is compatible with the data presented in Figure 11, which demonstrates that more of an absorbed dose enters the microcirculation when the rat-rat flap system is used.

A comparative analysis of the dose of caffeine absorbed is presented in Table 2.

CAFFEINE: 1 vs 2 DOSES

(36 μ g/cm²)

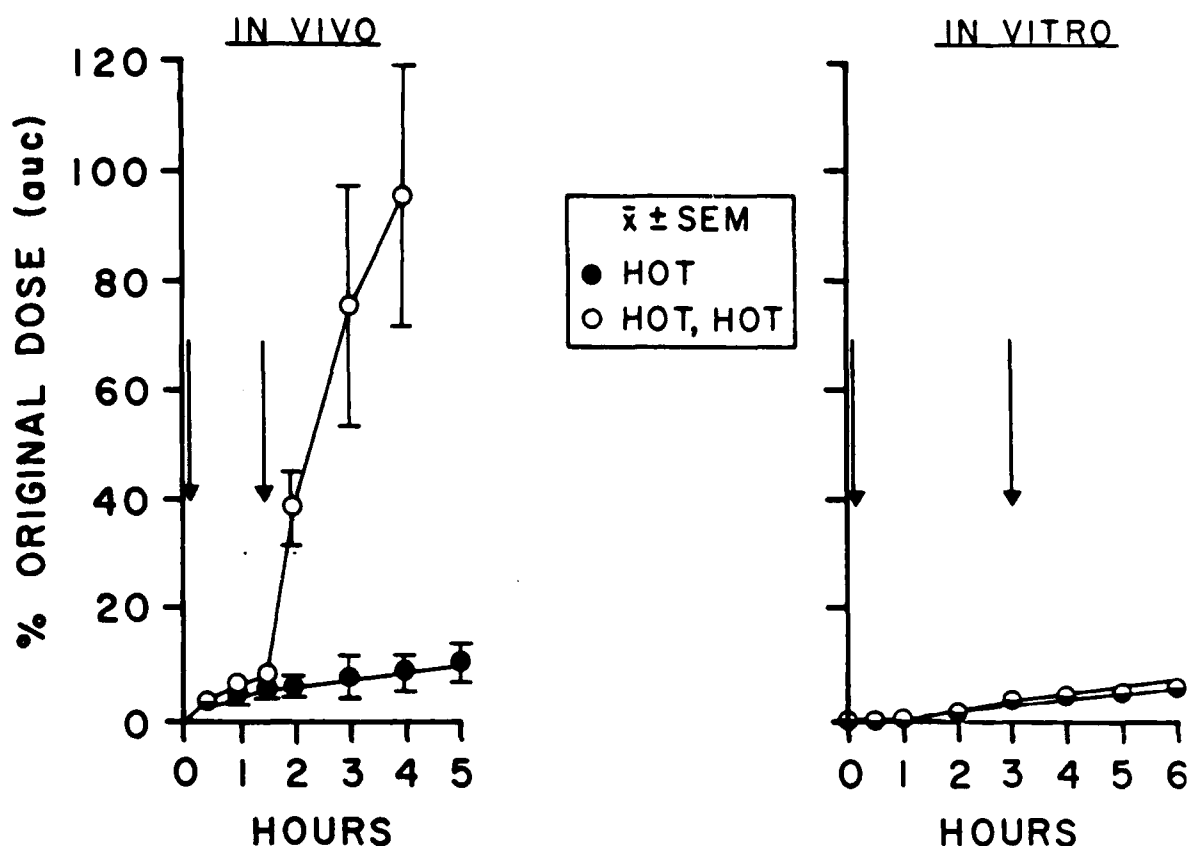


Figure 13. Comparison of Application of a Standard Dose of ¹⁴C-Caffeine. Comparison was made of application of ¹⁴C-caffeine in ethanol (see Methods) to human skin in vivo or in vitro. Less than expected amounts were noted in vitro; hence, a second dose was added at the time indicated by the arrow (both in vivo and in vitro). Both dosages were radioactive. In the in vivo setting, as a separate experiment, a second dose was added at 1.5 hours. This resulted in a prompt increase in the total amount of the original dose absorbed. A similar phenomenon was not observed in vitro.

TISSUE BINDING IN RAT/RAT
AND HUMAN/RAT SANDWICH FLAPS
FOLLOWING DEPOSITION OF
I ($36\mu\text{g}/\text{cm}^2$) DOSE CAFFEINE

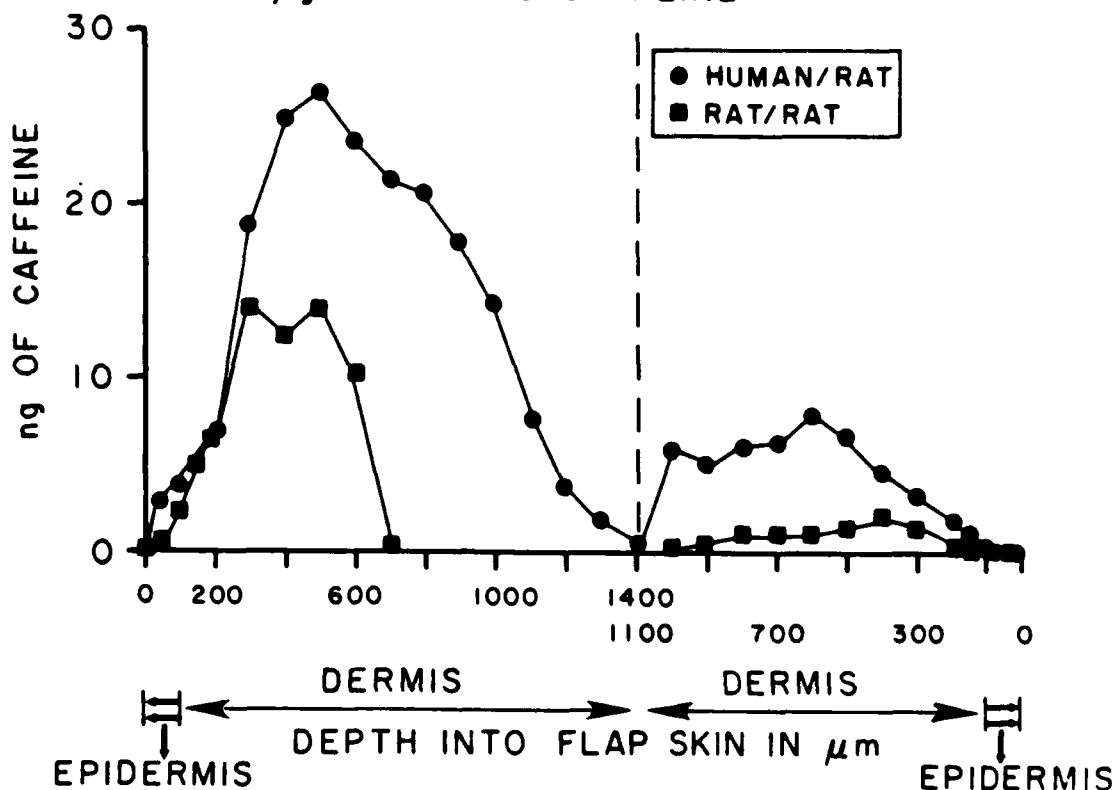


Figure 14. Analysis of Location of Binding within Rat and Human Skin Components of the Skin Sandwich Flap. This analysis was made following the topical deposition of ^{14}C -caffeine, using a standard dose ($36\mu\text{g}/\text{cm}^2$). Values on the vertical axis represent dosage of caffeine at that 50 and 100 micron interval.

Table 2
 Percutaneous Absorption of ^{14}C -caffeine in Ethanol Following Deposition
 of One and Two Doses on the Human Side of a Human-Rat Skin Flap in Vivo

# of Doses	Amount in Flap Blood at 4 Hours	Amount in Skin at 4 Hours
One	2.8 ug/cm^2 (9.4%) ^a	0.26 ug/cm^2 (0.9%)
Two ^b	22.0 ug/cm^2 (30.5%)	0.16 ug/cm^2 (0.2%)

^a Percent of original dose

^b 72 ug/cm^2

The increase in ^{14}C -caffeine absorbed into flap blood 4 hours after the second dose is 10-fold that seen after one dose (22 vs. 2.8 ug/cm^2 , respectively). The amount of drug within this flap following one or two doses is, however, very similar. The amount in the skin does not account for the increase in flap blood concentration. Thus, the data support the hypothesis that tissue binding of caffeine results in a nonlinear absorption of compounds (caffeine in this case), and reiterates the importance of in vivo analysis.

Assessment of feasibility of transplanting hair-bearing skin to the nude rat

This feasibility experiment has been dictated by the observation that an increased amount of a topically applied agent is absorbed when applied to skin that is relatively rich in hair follicles, rat skin vs. human skin (see above). While the number of terminal hairs in the nude rat is less than that of its littermates, it is at least 10-fold that of normal nonhair-bearing human skin. To date, there have been no descriptions of hair growth following transplantation of human split-thickness skin grafts to nude rats. However, we noted hair growth in split-thickness scalp skin grafts on nude rats treated with cyclosporine. This phenomenon was not observed in similar grafts on nude rats treated with Imuran and hydrocortisone or with anti-lymphocyte serum. Thus, a preliminary analysis, using orthotopically grafted 0.4 mm and 1.0 mm STSG-SS, was undertaken. The objective was to determine whether hair growth really did occur, and what role thickness of the graft played in determining the ultimate density of the hair growth.

In rats grafted with split-thickness scalp grafts, two stages of hair growth were noted: immediate and delayed. In the immediate stage (the first 6 weeks of engraftment), hair growth was quite dense and occurred in the apparent absence of papillae (see Figure 15). The hair in this growth phase could be removed with very gentle tugging, and spontaneously fell out at 6-8 weeks. In the delayed stage (occurring 2-3 months after engraftment), hair growth was less dense (approximately 10% of that of Figure 15), and this hair could only be removed with firm tugging. In the delayed stage, there was histologic evidence of formation of new papillae. The density of hair in the delayed stage correlated with the thickness of the scalp skin grafts. New hair appeared on an average of 1/ cm^2 /week in the thick (1 mm) grafts. By 10 weeks, the density was 7.9 hairs per cm^2 . In the thinner (0.4 mm) grafts, the density of hair at 10 weeks was 3.5 hairs per cm^2 , a significant difference. At 10



Figure 15. Photograph of Hair Growth in a Human Split-Thickness Skin Graft on a Nude Rat. This photograph illustrates hair growth 6 weeks following orthotopic engraftment of human scalp skin (1.0 mm thick) to the lateral thoracic cage of the nude rat. The average length of hair was 1.0 cm (see Methods).

weeks postgrafting, the hair in the thicker grafts had a mean length of 4.4 mm, vs. 1.7 mm in the thinner grafts, a significant difference ($p < 0.001$). The average diameter of the hair shaft was that of terminal hair, 0.05 mm at the various times tested. Unfortunately, funding ended prior to our being able to determine whether or not scalp skin could be utilized as donor skin for the flap system. However, the foregoing data suggest that skin bearing functional adnexal structures, i.e., grow hair, is feasible. Further, it is anticipated that differences can be made in percutaneous absorption of topically applied compounds between adnexal-rich and adnexal-poor skin.

Animal and experimental statistics

Statistics on the loss of sandwich flaps and the number of experiments performed per flap have been collected over the past year (Dec 84-Dec 85) to highlight problem areas in the generation of the flap in hopes of improving the survival rates. Data presented will be analyzed according to: 1) the total number of flaps generated per quarter (rat-rat and human-rat), 2) the total number of flaps lost to flap chewing and the stages during flap generation (I, II, III and >III), in which the chewing occurred, 3) the number of experiments per flap (1, 2, 3, 4, and >4), and 4) the total number of experiments performed per quarter. The two latter categories differentiate between experiments on flaps generated within that quarter and those that survive to be utilized experimentally at times outside that quarter. The number of experiments per quarter may therefore be less than the total number of experiments performed per flap.

The total number of flaps generated each quarter remains about the same over the four quarters, approximately 40 for each quarter: approximately 12 rat-rat flaps and approximately 27 human-rat flaps. The number of human-rat flaps generated depended upon our experimental needs and the availability of human skin from elective plastic surgery procedures. Skin availability has been relatively stable over the time analyzed.

The percent of rat-fat flaps generated that are lost to flap chewing has remained relatively stable at 50%. Flap chewing of the human-rat flaps has decreased from 42% in the second quarter to 27% in the fourth quarter. Flap chewing is a continuing problem that we have specifically addressed over the past few months and may account for the decrease to 27%. Regardless of the apparatus designed, the animal is still able to chew at its flap if it so desires. The chewing behavior is not predictable and is not necessarily related to graft rejection. Some animals go for prolonged periods of time without chewing at their flaps. For example, one animal, Z1 with a rat-rat flap, was used experimentally on 12 separate occasions without ever bothering its flap. The use of metal collars around the rat's neck prohibits chewing but also inhibits grooming behavior, which leads to illness and often death. The course of prevention we are presently pursuing involves more frequent changing of the restraining bandage, allowing time between bandaging for grooming. We continue to look at other systems which do not include tranquilizing drugs. We hesitate to use these drugs, since they may interfere with metabolism studies to be done in the future. Although we continue our search for new methods to inhibit the chewing behavior, the reality may be such that a certain percent of flap loss due to chewing may be inevitable. Our goal is to reduce that percentage as much as possible.

The percent of total rat-rat flaps generated which are lost to graft

rejection has decreased from about 25% in quarters I, II and III to 0% in quarter IV. Although this decrease is dramatic and ideal, the percent of graft rejection most probably will not be maintained at that level. The loss of human-rat flaps due to graft rejection has decreased slowly from 41% in the first quarter to 29% in the third quarter. Closer examination of the rejection data reveals that the majority of rejection in the first quarter occurred after stage III (15 animals). At that time, animals were receiving 20 mg/kg cyclosporine subcutaneously on day 1, and thereafter receiving 12.5 mg/kg cyclosporine subcutaneously every other day through day 21. Maintenance dosing consisted of 12.5 mg/kg subcutaneously once per week for the duration of flap survival. Realizing that the maintenance dose was clearly inadequate, we have altered the dosing regimen to 20 mg/kg cyclosporine subcutaneously on day 1, followed by 12.5 mg/kg subcutaneously every other day through day 14 and maintaining cyclosporine in the drinking water at a dose of 11 mg/kg/day. This new dosing regimen has shifted the major incidence of graft rejection in the human-rat flaps occurring in the >III stage of flap generation seen in the first and second quarters (Dec 84-Mar 85 and Apr 85-Jun 85) to earlier stages in the third and fourth quarters.

Flap losses in quarter IV due to graft rejection increased to 48% of the total human-rat flaps generated. The increase is reflective of the type of skin used for grafting. During the fourth quarter, we started grafting human face skin as well as human abdominal skin. Graft rejection of the human face skin has initially been much greater than that of abdominal skin grafts. Technical difficulties associated with grafting multiple pieces of face skin to the rat sandwich host skin has been circumvented by suturing small pieces of face skin together prior to grafting. Minimizing the losses of human-rat flaps from graft rejection in the early stages of generation is still a concern and thus a study is underway of the cyclosporine concentration in whole blood throughout the therapy with cyclosporine.

The most significant improvement in the flap statistics lies in the number of experiments performed per flap. In the first quarter, the majority of flaps were utilized in one experiment only (n=11). In the second, third, and fourth quarters, however, many animals, with both rat-rat and human-rat flaps, were utilized in two or more experiments. One particular rat-rat flap, which was generated in the second quarter, was used on 12 separate occasions over a period of 5 months. The statistics of the fourth quarter are incomplete, since some flaps are still viable and future experiments are expected to be performed. The average number of experiments performed per flap is approximately three for both rat-rat and human-rat flaps.

The final category, the total number of experiments per quarter, is reflective of the productivity of a two-person team involved in the generation, experimentation, and maintenance of the flaps. In general, 17 experiments have been performed over the fourth quarter, which is an average of 1.4 experiments/week. Of course, this is directly dependent upon the number of flaps available for experimentation, which varies from week to week. In the last quarter of the year (Oct 85- Dec 85), 27 experiments were performed. This is the result of experimentation on animal flaps generated in that specific quarter as well as previous quarters. The skin sandwich flap has become a functioning reality. We continue to improve our methodologies in the generation and maintenance of the sandwich flap and those improvement are revealed in the statistical data (see Table 3).

Table 3
Animal Statistics

DATE	TOT FLAPS	FLAPS CHEWED						GRAFT FAILURE						# EXP / FLAP						# EXP/TOT
		I	II	III	>III	T	%T	I	II	III	>III	T	%T	1	2	3	4	5	6	
<u>DEC 84-MAR 85</u>																				
rat/rat	4	0	0	1	1	2	50	0	0	0	1	1	25	0	0	0	0	0	0	0
human/rat	58	2	0	0	9	11	19	4	3	2	15	24	41	12	3	0	0	0	10	10
TOTAL	62	2	0	1	10	13	21	4	3	2	16	25	40	12	3	0	0	0	10	10
<u>APR 85-JUN 85</u>																				
rat/rat	16	2	0	1	5	8	50	1	3	0	0	4	25	2	2	0	1	1*	2*	0
human/rat	26	3	1	2	5	11	42	0	2	2	5	9	35	1	2	0	1	0	0	0
TOTAL	42	5	1	3	10	19	45	1	5	2	5	13	31	3	4	0	2	1*	2*	0
<u>JUL 85-SEP 85</u>																				
rat/rat	11	2	1	1	1	5	45	2	0	1	0	3	27	0	2	0	0	0	4	0
human/rat	24	1	5	3	0	9	38	2	0	4	1	7	29	3	1	1	0	1*	13	0
TOTAL	35	3	6	4	1	14	40	4	0	5	1	10	29	3	3	1	0	1*	17	0
<u>OCT 85-DEC 85</u>																				
rat/rat	11	2	2	2	1	7	64	0	0	0	0	0	0	2	1	0	0	1*	0	15
human/rat	30	3	2	2	1	8	27	3	10	0	4	17	57	2	1	0	0	0	4	10
TOTAL	41	5	4	4	2	15	37	3	10	0	4	17	41	4	2	0	0	1*	4	25

* 12 EXPERIMENTS

5 EXPERIMENTS

Conclusions

The foregoing, as well as progress listed in our second annual report, suggest that the human-rat skin sandwich flap is a viable system for assessing percutaneous absorption. While we have been unable to complete all of the validation experiments proposed, relative to a profile of absorption of various compounds, e.g., hydrocortisone, testosterone, and malathion, the data presented in this report confirm the feasibility of this system. Issues that will continue to be addressed include a determination of the effects of cyclosporine on percutaneous absorption, the influence on blood flow by the barrier function of the stratum corneum, determination of differences in rates of absorption of standard chemicals across adnexal structure-rich vs. adnexal structure-poor human skin, as well as a further determination of in vitro vs. in vivo analysis, comparing various standard chemicals. Such an analysis will permit a comparison with values obtained on human subjects prior to such times as concerns were generated relative to continuing to perform these types of studies on human volunteers.

To our knowledge, the human/rat skin sandwich flap is the first example of a viable, functional human organ that is chronically maintained by a biologic support system, which has the added distinction of being supplied by an independent but accessible vasculature. The foregoing experiments strongly suggest that this system will be important in gaining insights into the more sophisticated percutaneous absorption processes of human skin. Additional features of this system, which make it attractive as an experimental system, include: the ability to monitor systemic blood levels as a function of the levels of test compounds in the flap blood; the ability to monitor the disappearance of the test compound from the donor chamber, and therewith the ability to accurately determine transdermal flux.

Predicting the utility of this system is likely to be interpreted as presumptuous. However, it would appear that the system will be of value in:

- A. Studying the interaction of skin with toxic substances, e.g., what is the time from application to blood levels sufficient to cause systemic symptoms? Are protective creams/devices of value in decreasing the toxicity following the application of toxic substances to human skin?
- B. Studying the metabolic capacity of skin, relative to the rest of the organism, e.g., are prodrugs metabolized in the skin with sufficient speed to permit therapeutic blood levels? Does the skin metabolize compounds via a different pathway from the liver which makes the metabolites more or less useful for therapy?
- C. Studying skin as a barrier to drug delivery, e.g., what is the transdermal flux of the drug in question? Can this be altered in the in vivo setting with agents which enhance absorption?
- D. Studying blood flow and environmental factors, e.g., humidity and temperature, on percutaneous absorption and metabolism.
- E. Studying the delivery of systemic drugs to the skin, e.g., following the oral ingestion of an anti-fungal agent, how long is it before fungicidal levels are present in the stratum corneum?

- F. Studying the metabolism of natural hormones, cytokines, or drugs in the skin, e.g., can it be demonstrated that an intra-arterial injection of testosterone into the femoral artery supplying the flap results in metabolism of testosterone within the skin? Are the metabolic products different for different types of skin, such as scalp skin of males with androgenetic alopecia, vs. normal males.
- G. Studying inflammatory processes, e.g., will the injection of antisera with high titers of antibody to the basement membrane from patients with pemphigoid result in the generation of inflammatory mediators, such as components of the complement cascade in the venous blood draining the flap? Can cytokines direct lymphocyte trafficking to the epidermis?

These are but a few of the types of questions for which we believe the system will have utility. Only with continued experiments aimed at validating the system and challenging the system will we determine whether our current optimism for this system is justified.

Future Objectives

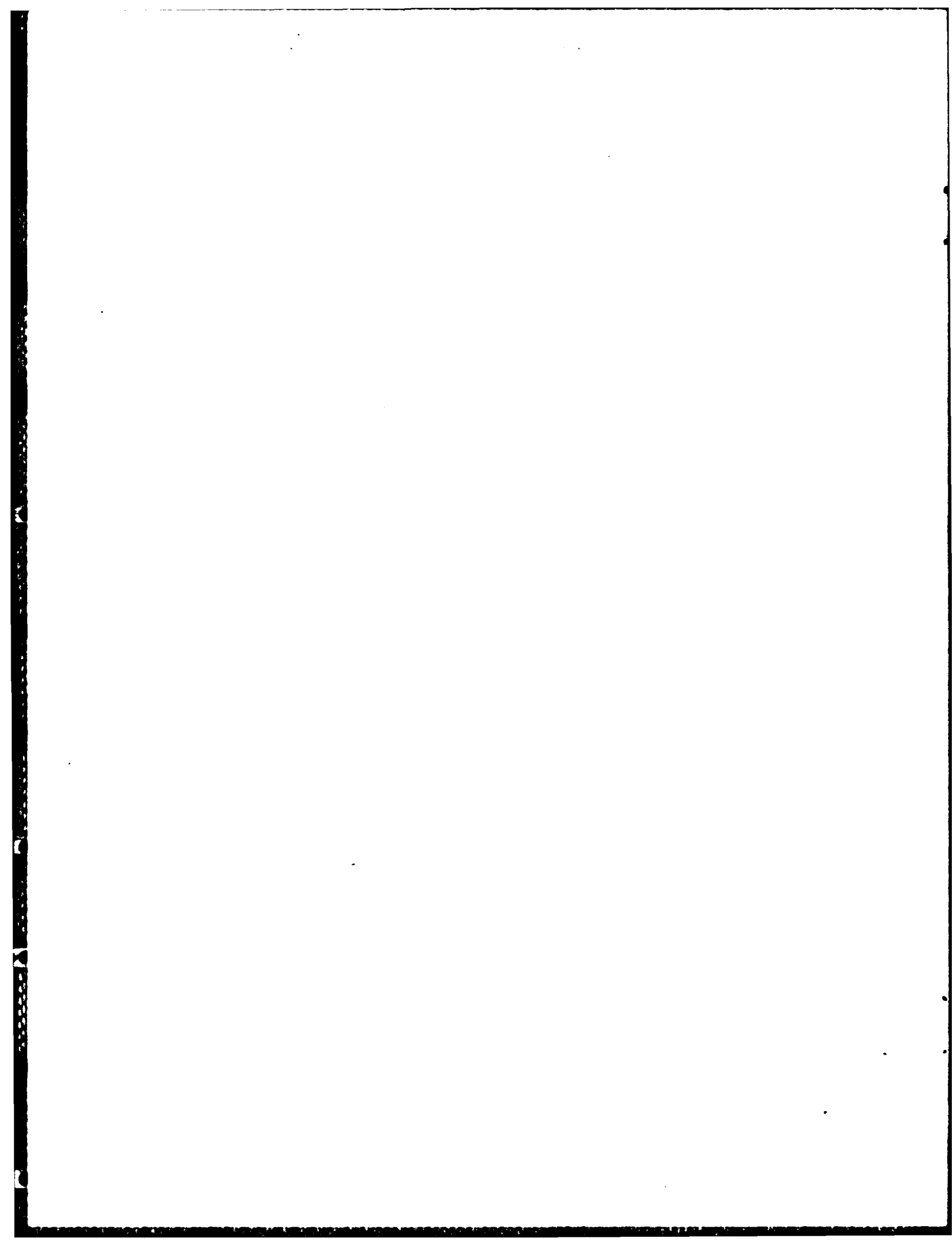
The funding requested for this project was for three years. This was supplemented with a three month extension. This annual report covers the last funding period; hence, future objectives are not appropriate at this juncture. Nevertheless, it is our plan to submit a new proposal which continues with these objectives, and approaches the important aspect of this model system in studying cutaneous toxicity, specifically with regard to human skin reacting to a series of compounds which are known to be toxic when applied percutaneously.

Literature Cited

1. Scheuplein RJ, Bronaugh RL: Percutaneous absorption, in: Biophysical Properties of the Skin, Vol. 1, LA Goldsmith (ed), New York, Oxford University Press, 1983, pp. 1255-1295.
2. Reifenrath WG, Chellquist EM, Shipwash EA, Jederberg WW, Krueger GG: Percutaneous penetration in the hairless dog, weanling pig, and grafted athymic nude mouse: Evaluation of models for predicting skin penetration in man. *Br J Dermatol (Suppl 27)* 111:123-135, 1984.
3. Krueger GG, Briggaman RA: The nude mouse in the biology and pathology of skin, In: *The Nude Mouse in Experimental and Clinical Research*, 2nd Edition, J Fogh, BC Giovanella (eds), New York, Academic Press, 1982, pp. 301-322.
4. Brooks CG, Webb PJ, Robins RA, Robinson G, Baldwin RW, Festing MRW: Studies on the immunobiology of rnu/rnu "nude" rats with congenital aplasia of the thymus. *Eur J Immunol* 10:58-67, 1980.
5. Vos JG, Kreeftenberg JG, Kruijt BC, Kruizinga W, Steerenberg PA: The athymic nude rat. II. Immunologic characteristics. *Clin Immunol Immunopathol* 15:229-236, 1980.
6. Marks NJ: Quantitative analysis of skin flap blood flow in the rat using laser Doppler velocimetry. *J Roy Soc Med* 78:308-312, 1985.
7. Holloway EA, Watkins DW: Laser Doppler measurement of cutaneous blood flow. *J Invest Dermatol* 69:306-309, 1977.
8. Watkins DW, Holloway GA: An instrument to measure cutaneous blood flow using the Doppler shift of laser light. *I.F.F.F. Trans Biomed Eng* 25:28-38, 1978.
9. Silverman DG, La Rossa DD, Barlor CH, et al: Quantification of tissue fluorescein delivery and prediction of flap viability with the fiberoptic dermofluorometer. *Plast Reconstr Surg* 66:545-551, 1980.
10. Graham GH, Walton RL, Elings VB, Lewis FR: Surface quantification of injected fluorescein as a predictor of flap viability. *Plast Reconstr Surg* 71:826-832, 1983.
11. Reinisch JF: The pathophysiology of skin flap circulation; the delay phenomenon. *Plast Reconstr Surg* 54:585-598, 1974.
12. Marks NJ, Trachy RE, Cummings CW: Dynamic variations in blood flow as measured by laser Doppler velocimetry: A study in rat skin flap. *Plast Reconstr Surg* 73:804-810, 1984.
13. Petry JJ, Wortham KA: The anatomy of the epigastric flap in the experimental rat. *Plast Reconstr Surg* 74:410-415, 1984.
14. Finseth F, Cutting G: An experimental neurovascular island skin flap for study of the delay phenomenon. *Plast Reconstr Surg* 61:412-420, 1978.
15. Sasaki GH, Pang CY: Hemodynamics and viability of acute neurovascular island skin flaps in rats. *Plast Reconstr Surg* 65:152-159, 1980.
16. Scheuplein RJ, Ross LW: Mechanism of percutaneous absorption vs. percutaneous absorption of solvent-deposited solids. *J Invest Dermatol* 62:353-360, 1974.
17. Feldmann RJ, Maibach HI: Absorption of some organic compounds through the skin in man. *J Invest Dermatol* 54:390-394, 1970.
18. Franz TJ: Percutaneous absorption. On the relevance of in vitro data. *J Invest Dermatol* 64:190-195, 1975.
19. Headington JT: Transverse microscopic anatomy of the human scalp: A basis for a morphometric approach to disorders of the hair follicles. *Arch Dermatol* 120:449-456, 1984.

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